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PATENT
Our Docket: SALK2190

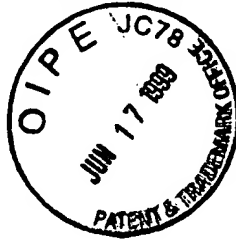
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
O'Gorman et al.

Serial No.: 08/919,501

Filed: August 28, 1997

For: SITE-SPECIFIC RECOMBINATION
IN EUKARYOTES AND
CONSTRUCTS USEFUL
THEREFOR



) Group Art Unit: 1633

) Examiner: M. Wilson

) CERTIFICATION UNDER 37 CFR §1.8

) I hereby certify that the documents referred to as enclosed herein
) are being deposited with the United States Postal Service as first
) class mail on this date, 6/14, 1999 in an envelope
) addressed to: Assistant Commissioner for Patents, Washington,
) D.C. 20231

) Stephen E. Reiter, Reg. No. 31,192

) (Name of person mailing paper)

) Signature E. L. 6/14/99
) Date

Assistant Commissioner for Patents
Washington, D.C. 20231

DECLARATION OF
APPLICANT UNDER 37 C.F.R. §131

Sir:

We, Stephen O'Gorman and Geoffrey Wahl, the joint inventors of the above-identified application, do hereby declare and state that:

We are familiar with the content of the above-identified application.

We are aware that claims 1, 2, and 4 of the above-identified application have been rejected for alleged anticipation under 35 U.S.C. §102 over Lewandoski *et al.* (*Current Biology* 7:148-151, 1997) (hereinafter "Lewandoski") and claims 1-2, 4-5, 10-16, 18-19 and 24-44 have been rejected for alleged obviousness under 35 U.S.C. §103(a) over a combination of references including Lewandoski.

Best Available Copy

The claimed invention was conceived and reduced to actual practice by us in the United States as joint inventors prior to the publication date of the Lewandoski article, as supported by the evidence which follows:

At the time the present invention was conceived and reduced to actual practice, Stephen O'Gorman was the leader of the research team that performed the experiments set forth as Examples 1 through 5 of the above patent application, which experiments were completed in laboratories of The Salk Institute for Biological Studies in La Jolla, California prior to the publication date of the Lewandoski article. In support of this statement are attached true copies (with only the dates redacted) of pages from laboratory notebooks belonging to The Salk Institute for Biological Studies, as follows:

1. A 652 bp fragment of the mP1 promoter was isolated by PCR using genomic DNA from CCE embryonic stem cells as a template (Lab notebook pages 1 through 5).
2. The mP1 promoter fragment was fused to a modified Cre coding sequence in the plasmid pOG304M (Lab notebook pages 6 through 10).
3. A Cre expression plasmid pOG231 was prepared by fusing a modified Cre coding sequence to a synthetic intron and CMV promoter. (Lab notebook pages 11 through 13).
4. A plasmid pOG277 containing a loxP-flanked neomycin cassette was prepared by inserting a wild-type loxP site into pBSKS (Stratagene) and then cloning a neomycin cassette between interations of this loxP site. (Lab notebook pages 14 through 16).
5. A *hoxb-1* targeting construct containing a *NruI* site was prepared and the loxP-flanked neo cassette from pOG277 was inserted into the *NruI* site. (Lab notebook pages 17 through 29).

6. The P2Bc allele of Figure 1 was created by inserting a Lox-P flanked neomycin cassette and a β -GAL sequence into RNA polymerase II. (Lab notebook pages 45 through 61).
7. Transgenic mice were obtained from fertilized mouse oocytes injected with the protamine-Cre fusion gene from pOG304M (Lab notebook pages 62 through 65).
8. Heterozygous ProCre males were mated to wild-type females and the resulting progeny were examined by Southern blotting to determine the segregation pattern of ProCre nucleic acid constructs and P2Br alleles. (Lab notebook pages 30 through 44 and 62 through 67).

Thus, we maintain that the subject matter contained in the above-identified application was conceived and reduced to actual practice by us prior to the date of publication of the Lewandoski reference.

We further declare that all statements made herein of knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine, or imprisonment, or both under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

6/14/99
Date

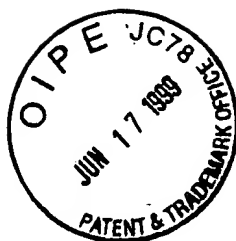
Stephen O'Gorman
Stephen O'Gorman

6/14/99
Date

Geoffrey Wahl
Geoffrey Wahl

Attachments: Laboratory Notebook Pages 1-67.

Protamine promoter PCR



FILENAME: PROTAMINE PROMOTER PRIMERS
IN: LAB/SEQUENCES/CRE/PROTAMINE

This is a pair of primers to amplify the mouse protamine 1 promoter from -560 to the translational start. The 5' primer includes a Bgl II site. The 3' primer contains a SmaI site. Both of these sites are meant to allow us to drop the PCR product into pOG234. The PCR product should be about 570 bp in length.

Primer positions: U 1 L 630

Upper/Lower: the most stable 3'-dimer: 2 bp, -1.3 kcal/mol

5' GAAGATCTGTCTAGTAATGTCCAACACCTCCCTCAGT 3' → **GMW 310**

3' GAGACTCGGTCGAGGGCCGGTTCGGTCGTGGGCCCATC 5'

Upper/Lower: the most stable 3'-dimer: 3 bp, -3.2 kcal/mol

5' GAAGATCTGTCTAGTAATGTCCAACACCTCCCTCAGT 3'

3' GAGACTCGGTCGAGGGCCGGTTCGGTCGTGGGCCCA

Upper/Lower: the most stable dimer overall: 5 bp, -9.4 kcal/mol

5' GAAGATCTGTCTAGTAATGTCCAACACCTCCCTCAGT 3'

3' GAGACTCGGTCGAGGGCCGGTTCGGTCGTGGGCCCATC 5'

Lower Primer, 38-mer [630]:

Lower Primer: the most stable 3'-dimer: 3 bp, -3.2 kcal/mol

5' CTACCCGGGTGCTGGCTTGGCCGGGAGCTGGCTCAGAG 3' → **GMW 311**

3' GAGACTCGGTCGAGGGCCGGTTCGGTCGTGGG

Lower Primer: the most stable dimer overall: 8 bp, -18.6 kcal/mol

5' CTACCCGGGTGCTGGCTTGGCCGGGAGCTGGCTCAGAG

3' GAGACTCGGTCGAGGGCCGGTTCGGTCGTGGGCCCATC 5'

Hairpin: $\Delta G = -8.0$ kcal/mol, Loop = 12 nt, $T_m = 98^\circ$

5' CTACCCGGGTGCTG

3' GAGACTCGGTCGAGGGCCGGTTCG

Protamine promoter PCR

Upper Primer, 37-mer [1]:

Upper Primer: the most stable 3'-dimer: 2 bp, -1.3 kcal/mol

5' GAAGATCTGTCTAGTAATGTCCAACACCTCCCTCAGT 3'

3' TGACTCCCTCCACAACCTGTAATGATCTGTCTAGAAG 5'

Upper Primer: the most stable dimer overall: 6 bp, -7.9 kcal/mol

5' GAAGATCTGTCTAGTAATGTCCAACACCTCCCTCAGT

3' TGACTCCCTCCACAACCTGTAATGATCTGTCTAGAAG 5'

Hairpin: $\Delta G = 1.3$ kcal/mol, Loop = 4 nt

5' GAAGATC

3' TGACTCCCTCCACAACCTGTAATGATCTGT

Optimal Annealing Temperature: 61.3°

Product length, GC content & Tm: 667 bp, 52.5% GC, 80.4°

Product Tm - Upper Primer Tm: 13.7°

Primers Tm difference: 17.5°

Upper Primer: 37-mer, pos. 1, Tm = 66.7°, 3'-pentamer $\Delta G = -6.4$ kcal/mol

Lower Primer: 38-mer, pos. 630, Tm = 84.1°, 3'-pentamer $\Delta G = -6.7$ kcal/mol

Salt & DNA Concentrations (fixed in this option): 50 mM and 250 pM, respectively

NOFILE

this is a sequencing primer for the protamine promoter. It is from the top strand of the promoter starting at 130 bp from the protamine translation initiation codon and extending for 20 bp.

It is oligo 319

Current Oligo, 20-mer [534]:

Current+ Oligo: the most stable 3'-dimer: 3 bp, -3.2 kcal/mol

```
5' GAGGAAGAGGGTGCTGGCTC 3'
   ||| ||| ||| |||
3' CTCGGTCGTGGGAGAAGGAG 5'
```

Current- Oligo: the most stable 3'-dimer: 3 bp, -3.2 kcal/mol

```
5' GAGCCAGCACCTCTTCCTC 3'
   ||| ||| ||| |||
3' CTCCTTCTCCACGACCGAG 5'
```

Current+ Oligo: the most stable dimer overall: 3 bp, -3.2 kcal/mol

```
5' GAGGAAGAGGGTGCTGGCTC 3'
   ||| ||| ||| |||
3' CTCGGTCGTGGGAGAAGGAG 5'
```

Hairpin: $\Delta G = 0.9$ kcal/mol, Loop = 8 nt

```
5' GAGGAAGAGGGTG
   |||
3' CTCGGTC
```

FOR REACTIONS TO RUN ON PROTAMINE PROMOTER

0

Primers 310
311

Resuspend in 200 μ TE
Read 260/280 on 1:100 dilution
Concentration = 2X A260

A320	A280	A260	280/260	260/280	
0.0047	0.5788	1.0482	0.5501	1.8177	310
0.0090	0.7395	1.3386	0.5494	1.8202	311

Primer mix

310: $108 \div 2.1 \text{ g/l} = 4.8 \text{ l}$

311: $108 \div 2.7 \text{ g/l} = 3.7 \text{ l}$

91.5 μ H₂O to 100 μ

Reactions

Want to use ~ 18 μ CCF DNA/reaction, have stock that is 0.66 μ /l

Want to run 8 reactions

Mix for 10 reactions

(5) μ DNA

235 μ TE

Boil DNA 5 minutes

Place on ice immediately

THESE REACTIONS WERE USED IN THE CONSTRUCTION

OF PLASMIDS 304 and 305

GOOD PRODUCT OBTAINED (SEE NEXT PAGE)

0/4

PCR Reactions to Clone Proteinase Promoter

#	DNA	MgCl ₂	50mM NTP	100ng/l Primers	H ₂ O	2x PCR
1	25μl	2	2	1	28	50μl
2	↓	2	2	3	18	↓
3	↓	2	2	5	16	↓
4	↓	1	1	3	20	↓
5	↓	3	3	3	16	↓
6	↓	4	4	3	15	↓
7	↓	5	5	3	12	↓
8	↓	3	3	0	15	↓

- ① Load tubes and place on ice
- ② Add 2x PCR
DNA
Mg²⁺ NTP
Primers
- ③ Add 0.51 Tag polymerase
- ④ Mix by vortexing

Cycling Parameters

1x 94°C, 60 sec

35x 92°C, 30 sec

55°C, 1 min

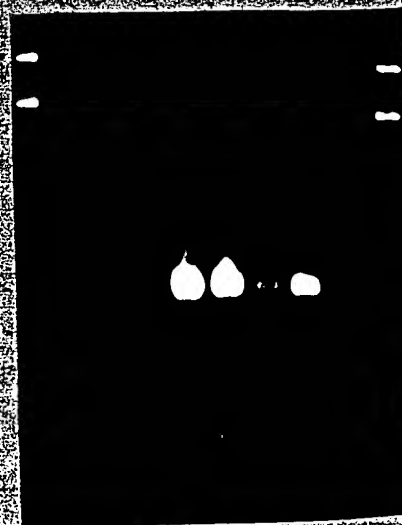
72°C, 72°C

5/12/95 10x each reaction run on a standard agarose.

#9 = best

Polymers mixed by the relative amount of product in the various lanes. There is no good reason why #4-6 should show lots of product and lanes 1-3 should show none.

It is possible that #9 should be the clearest reaction (1μM MgCl₂).



① Clean up and prep PCR reaction #4, product.

- add 120 μ l TE
- extract with 200 μ l p/c
- add 20 μ l 3M NaOAc
- add 1 μ l glycogen
- add 300 μ l EtOH
- 15 minutes ice
- 30 minutes TOMY
- wash pellet 70%
- resuspend in 20 μ l

② Digest PCR product with BglII and PspAI

PCR #4 only.

- 20 μ l DNA
- 20 μ l 10x #3
- 10 μ l PspAI
- 10 μ l BglII
- 140 μ l H₂O

③ Digest pCG234 with BglII and SmaI

- 7.5 μ l DNA = 38 (Q.P. at 0.4 μ l/ μ l)
- 20 μ l 10x Sma
- 10 μ l SmaI
- 10 μ l BglII
- 152.5 μ l H₂O to 200 μ l

④ Digest pCG234 with PspAI and SmaI

- 7.5 μ l DNA = 38
- 20 μ l 10x Sma
- 10 μ l SmaI
- 10 μ l PspAI
- 152.5 μ l H₂O to 200 μ l

⑤ Digest pCG231 with EcoRI + Klenow

- 1.7 μ l DNA = 38 (prep at 1.8 μ l/ μ l)
- 10 μ l 10x Eco
- 5 μ l EcoRI
- 83.3 μ l H₂O to 100 μ l

one hour
+ 4 μ l 2mM dNTP's
+ 2 μ l Klenow

30 min RT
65°C 5 min
bring to 200 μ l

Extract and digest with p/c

⑥ Digest PCR reaction #5 with SmaI (RT)

- 10x DNA
- 20x 10x #3
- 20x SmaI
- 150x H₂O

Extracted all 5 digests 1x with p/c, precipitated.
resuspended in 20x TE

⑦ Digest PCR reaction #6 with SmaI

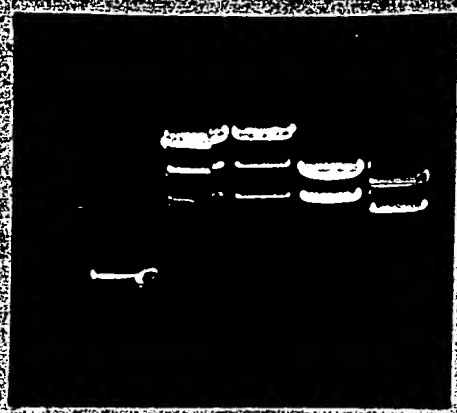
- 2x DNA
- 5x 10x #3
- 5x SmaI
- 20x H₂O

⑧ Digest PCR reaction #7 with SmaI

- 2x DNA
- 5x 10x #3
- 5x SmaI
- 20x H₂O

⑨ Digest PCR reaction #8 with SmaI

- 10.5x DNA
- 3x 10x #3
- 3x SmaI
- 2x H₂O
- 20.5x H₂O



LMA gel

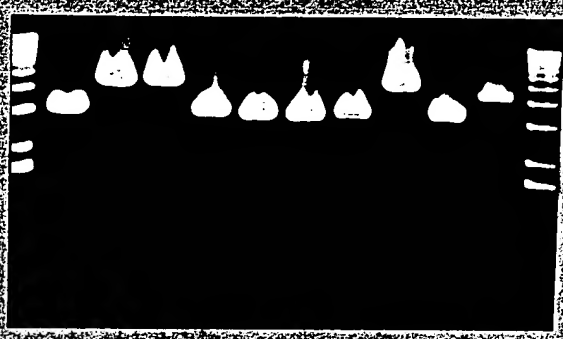
- ① PCR with Bgl I and Pst I
- ② ③ 234 with Pst I + SQT
- ④ ⑤ 234 with Bgl II + SQT

304 U1 7.5A PCR gel
7.5A 234 18kb
5A 234 vector

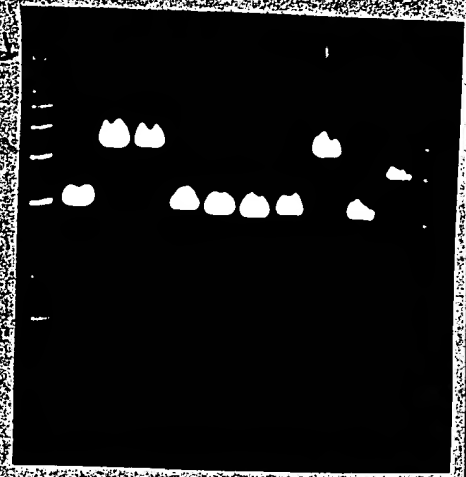
304 U2 15A Blank
5A 234 vector

Transformed into PG-1
Good transformation ratio

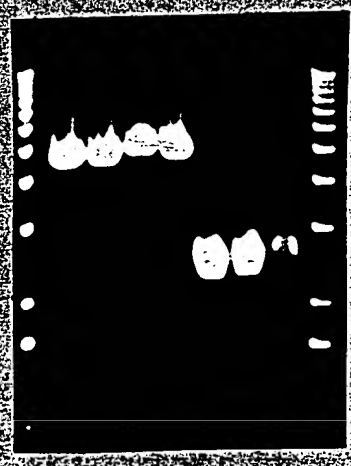
12 colonies digest with Pst I #2,3 look good



RL#2,3 saved



further checking digest RL#2,3



K

304 RL#2 & #3
Sma I
Bgl II
Bgl II + Hind III
234 (100ng) Bgl II + Hind III

②

Digest 105001 w/ SmaI and PstI (want ~ 3kb band)

0.6 μ DNA (10) Cat 19.111

5 μ 10x #4

4 μ SmaI

1 μ PstI

40 μ H₂O (to 50 μ)

15.35 \rightarrow 0.6% LMA gel

Digest 105306 w/ SmaI and PstI (want 1600bp band)

0.5 μ DNA (10)

5 μ 10x #4

4 μ SmaI

1 μ PstI

40 μ H₂O (to 50 μ)

20.35 \rightarrow 0.6% gel

Ligations

L1 5 μ 304 gel

5 μ 306 gel

12 μ 10x gel

1 μ ATP

1 μ T4 ligase

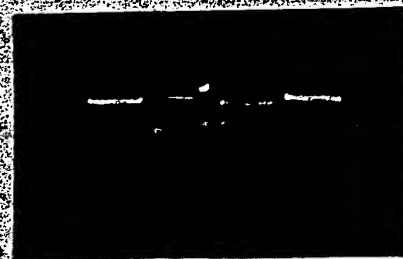
86 μ 10 mM Tris

L2 =

15 μ blank

304

306



14°C O/N

Ligations transformed

Slightly more w/ L2 than L1 to phage

Rapids digested w/ Sma & Pst w/ 10 μ run on 1% gel



~ 11 used for QP

Do checking digests of 304 M

#3 ① Bgl II

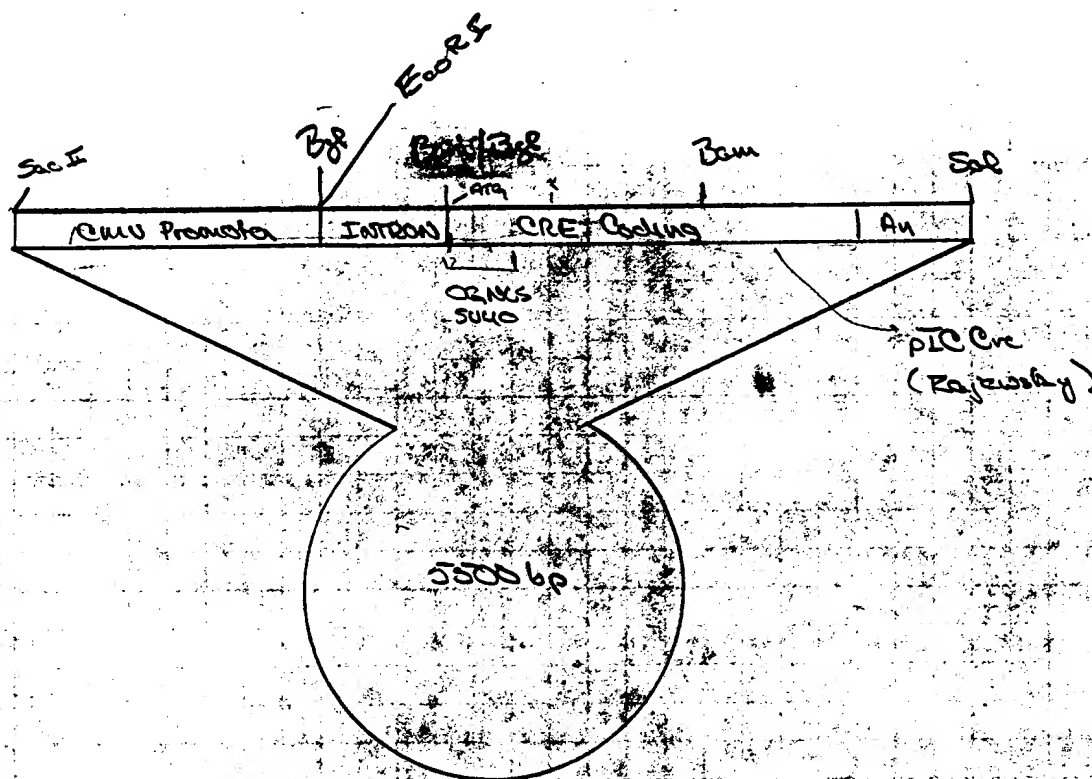
vac. #4 ② Sma I

sal ③ Sal I

④ Bgl II + Sal I (Sal buffer)

Drogen Prep.





Vector = pCG49 ΔBB cut with Bgl II and Sal I

Insert = pCG234 cut with Bgl II and Sal I

① Digest 234 with Bgl II

DNA - 30λ
 10x3 20λ
 Bgl II 20λ
 H₂O 130λ

All three preps digested one hour at 37°C. Then extracted with phenol/chloroform and precipitated.

② Digest 44ΔB with Bgl II

DNA 25λ (25λ)
 10x3 10λ
 Bgl II 7λ
 H₂O 80λ

DNA spun down, washed

234 up in 30λ
 44ΔB up in 25λ } 1λ each into mini
 ΔBB up in 30λ

③ ~~Digest 44ΔBB with Bgl II~~

~~DNA 2λ (3.28)
 10x3 10λ
 Bgl II 7λ
 H₂O 61λ~~

SHOULD HAVE
 CUT ΔBB with
BAM



Sal I digests

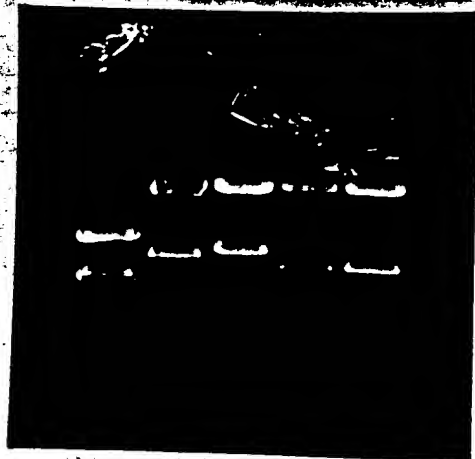
234 DNA 30λ
 H₂O 10λ

2B DNA 25λ
 H₂O 15λ

ΔBB DNA 30λ
 H₂O 10λ

all + 5λ Sal buffer
 5λ Sal
 one hour at 37°C
 2λ → minigel
 balance → LMA gel

From ΔB expect
 3911 + 2011
 From ΔBB expect
 3911 +



LMA gel (0.6% LMA in TAE)

- 1) 234, lower band excised
- 2) 44ΔB, upper band excised.

Ligations

#1 15λ 234 gel
 5λ 44ΔB gel

#2 15λ blank gel
 5λ 44ΔB gel

+ 86λ (10μM) m
 12λ 10x gel
 1λ ATP
 1λ ligase

to 14°C @ 10PM

3/12

Transformations: 51 each mix into AS-1

Excellent transformation ratio obtained.

Colonies: picked for rapid lysates.

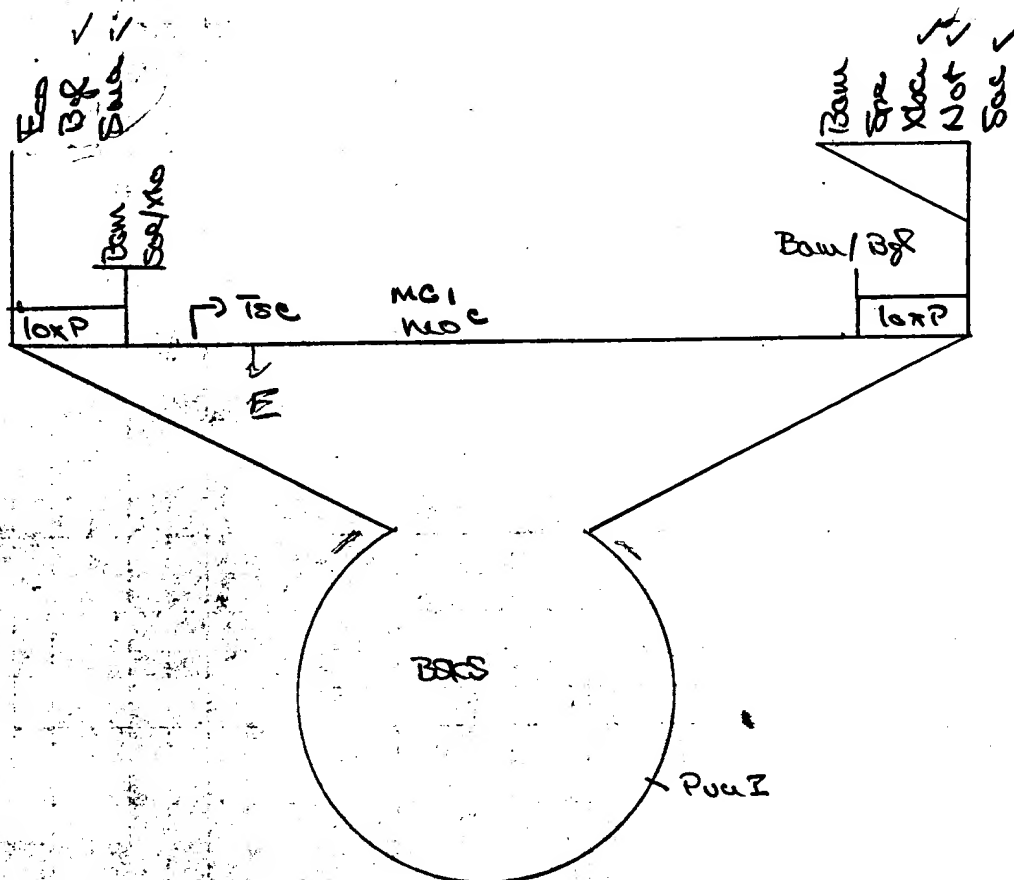


Std rapid lysates, $U_i = 50\text{A}$, 1d for repeats
 Sae + Bcm Digests. All give expected patterns

#1 & 2 saved

#1 needed for Qagen

$\checkmark = \text{unique}$



PvuI to BglI from pOR249B = 1200bp

PvuI to SacI from pOR249S = 1659bp

XhoI to BamHI from pMCIwobgA = 1133bp

① 1st digest 249B (RL#4) and 249S (RL#1) with ScaI

20 μ DNA
10 μ ScaI
20 μ 10x #3
150 μ H₂O

5 μ \rightarrow minigel
p/c extract
EtOH ppt with glycogen and NaOAc o/n
Wash pellet
Resuspend w/ 20 μ TE

② 2nd digest 249B with BglII

20 μ DNA
5 μ 10x #3
5 μ BglII
20 μ H₂O

5 μ \rightarrow minigel
15 μ and 35 μ \rightarrow 0.6% TAE
minigel.
Run 2 hours

③ 2nd digest 249S with ScaI

20 μ DNA
5 μ 10x ScaI
5 μ ScaI
20 μ H₂O

249B ScaI/BglII 5 μ
249S ScaI/ScaI 5 μ

LM A gel

249B 249S
15 μ 35 μ 15 μ 35 μ



Excised bands.

249B: ~1200bp band (lower)
249S: ~1700bp band (upper)

Ligations

277L1 5 μ 249B gel
5 μ 249S gel
10 μ MclV gel (7111)

277L2 5 μ 249B gel ()
5 μ 249S gel ()
10 μ blank gel

+ 86 μ 10 mM Tris 7.4
12 μ 10x gel ligation
1 μ 100 mM ATP
1 μ T4 ligase

-14 $^{\circ}$ O/N -

5' digestion used to transform 502 AGC

no colonies

5' ligation \rightarrow 100% AG-1

Good colony ratio, low but OK #'s

plates left at 37° for 48 hr

12 "areas" picked into 5 ml LB + Amp.

Repick: 5 cultures grown up, each digested
with SmaI and with NotI

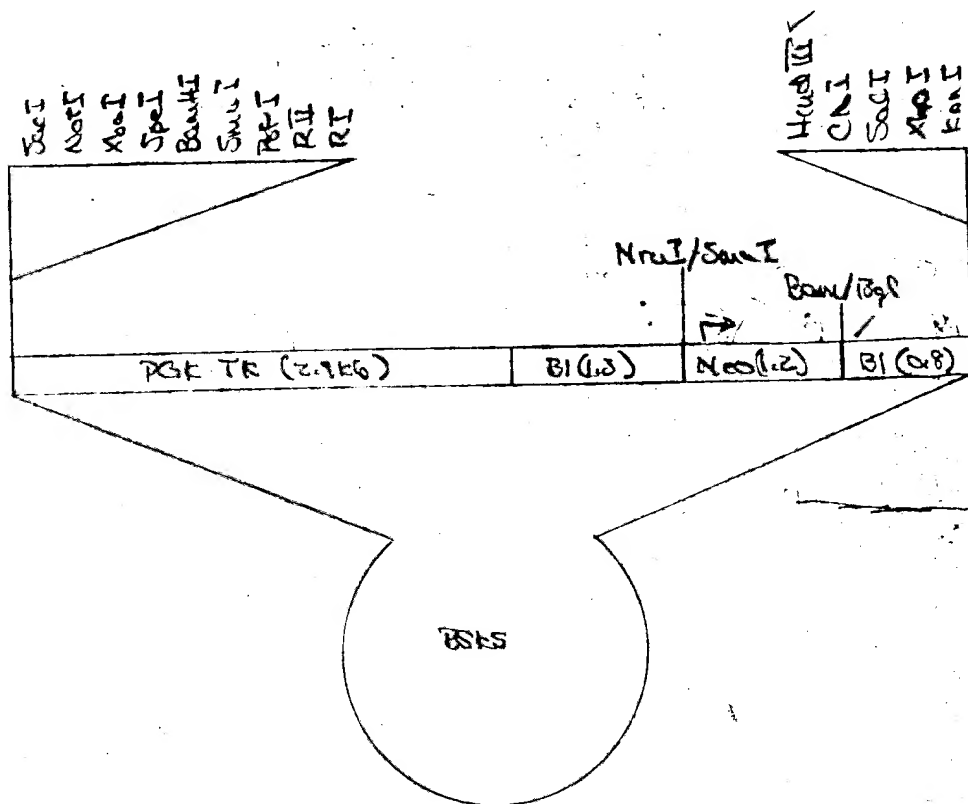


1-5 SmaI
1-5 NotI

#1 & 2 look good

#1 used for 293 transfection
test for recombination

#1 used for Qagen prep.



- 1) 336 NruI partial + HindIII
- 2) 336 BglII + HindIII
- 3) 277 BamHI partial + SmaI

4.2	1.2	3.7	EcoRI
4.1	4.9		BamHI
1.0	2.4	5.5	SacI
			NotI
			XbaI
			KpnI
			HindIII

① Digest 38 p06 336 with Nru I

1.6 μ l 336 (12/5/95 prep at 1.98/ μ l)
 20 μ l 10X Nru I
 5 μ l Nru I
 173.4 μ l H₂O
200 μ l

② Digest 108 p06 313 with Nru I

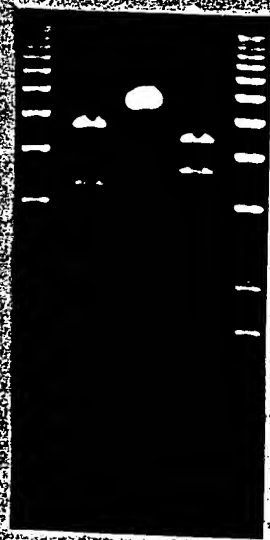
5.6 μ l 313 (8/30 prep at 1.88/ μ l)
 20 μ l 10X Nru I
 10 μ l Nru I
 164.4 μ l H₂O
200 μ l

③ Digest 58 p06 336 with Eco RI

2.6 μ l 336
 20 μ l 10X Eco
 310 μ l Eco RI
 167.4 μ l H₂O
200 μ l

One hour at 37°C

Run 7 μ l ①, 2 μ l ②, 4 μ l ③ on a minigel.
 Plc, ppt all three overnight.



Nru I digest of 336
 showed unmapped
 site in Tk. Will
 need to do a subcl

336 Nru I 7 μ l
 313 Nru I 2 μ l
 336 Eco RI 4 μ l

336 (100ng) Nru I
 Not I
 Kpn I
 342 (100ng) Kpn I

④

① 10x 336 cut with Not I $V_r = 200$ w/10x enzyme

5.2 λ DNA (2/5/95 prep at 1.98/ λ)
 20 λ 10x #3
 10 λ Not I
 164.8 λ H₂O

One hour at 37°C
 2 λ → minigel
 plc, ppt, spin, 70% EtOH wash,
 resuspend in 20 λ TE.

336 Not I

② Cut EcoRI digested 336 with Not I
 50% of resuspended DNA, 50 λ V_r 5x enzyme

All (my mistake) 20 λ DNA
 5 λ 10x #3
 5 λ Not I
 30 λ H₂O

One hour at 37°C
 1.5 λ → minigel

③ Partial EcoRI digest of Nru-cut 313

All of resuspended DNA
 $V_r = 200$ λ
 0.5 λ EcoRI
 3, 6, 12, 24, 45 minutes
 run 4 λ aliquots on ducking gel

20 λ DNA
 20 λ 10x Eco
 5 λ EcoRI
 159.5 λ H₂O

④ Partial NruI digest 336 (Not I) with NruI

All of resuspended DNA
 $V_r = 200$ λ
 0.5 λ NruI
 3, 6, 12, 24, 45 minutes
 run 4 λ aliquots on minigel

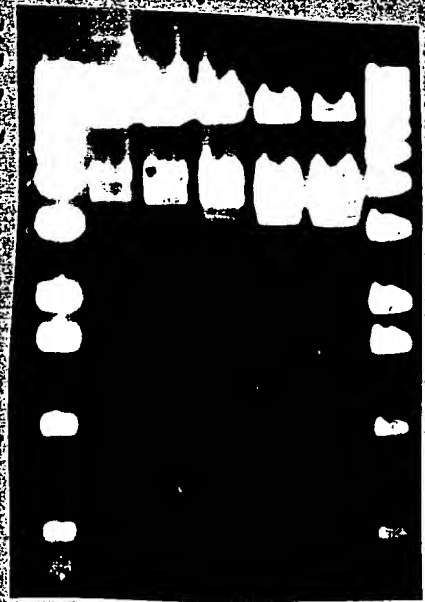
20 λ DNA
 20 λ 10x Nru
 0.5 λ NruI
 159.5 λ H₂O

313 partials

0.6% LMA/TAE gel

kb ladder

- ① 313 partial 6 min
- ② 30 λ 336 EcoRI/Not I
- ③ 336 partial 3 min
- ④ " 6 "
- ⑤ " 12 "
- ⑥ " 24 "
- ⑦ " 45 "



Min excised bands and
set up ligations.



Ligations L1 and L2 were transformed into
100% of lab stock AG15.

Transformation plates show about equal numbers
of colonies on L1 and L2.

Picked 18 colonies for rapid's tomorrow.

Standard Rapid lysates done - all 18 grew up.

Set up EcoRI digests



① Nru I partial digestion of 258 pOG 336

13 λ DNA (12/5/95 prep at 1.9 λ /1)
 20 λ 10X Nru
 0.5 λ Nru I
166.5 λ H₂O
 200 λ

To 37°C, timepoints taken at 3, 6, 12, 24, and 45 minutes.
 (40 λ \rightarrow 4 λ 250mM EDTA \rightarrow ice)

1 λ (125 μ g) of each timepoint ran out on a minigel.

Plc the rest of each timepoint and leave to ppt. overnight.

② Sma I + Spe I digest of pOG 277

5 λ DNA (7/30/94 prep at 1.8 λ /1)
 20 λ 10X #4
 5 λ Sma I
 5 λ Spe I
165 λ H₂O
 200 λ

Added 5 λ Sma I \rightarrow RT for 30 min.
 Added 5 λ Spe I \rightarrow 37°C for 30 min.
 4 λ \rightarrow minigel \rightarrow
 Plc, ppt. overnight.



DNA was spun down for 30 min., washed with 70% EtOH,
 respun and resuspended in 20 λ TE.

Redo Spe I digest of Sma I + Spe I digested 277

20 λ DNA
 20 λ 10X #2
 20 λ Spe I
140 λ H₂O
 200 λ

One hour at 37°C
 4 λ \rightarrow minigel

+ 8 λ 2mM each dNTP
 4 λ Klenow

30 min. at RT
 70°C for 5 min.

Plc, ppt.

- CIP 336 NruI cut DNA 45 timepoint

20 λ resuspended 336 DNA

5 λ 10 \times #2

3 λ CIP

22 λ H₂O

37°C 30', 15, 35 λ \rightarrow 0.6% LMA gel

resuspend 277 SmaI + SpeI + Klenow DNA

in 20 λ TE onto one lane of LMA gel

Ligations

L1 5 λ 334 gel

15 λ 277 gel

L2

15 λ blank

both

86 λ 10 mM Tris

12 λ 10 \times gel buff

1 λ ATP 100 mM

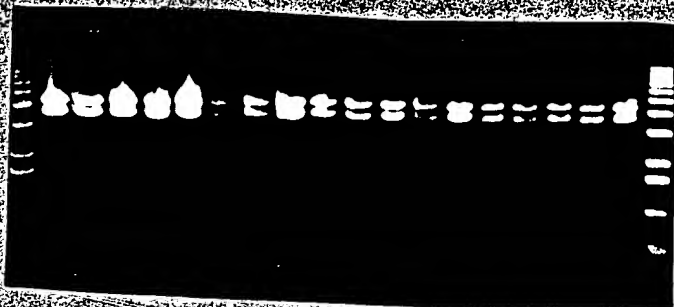
1 λ T4 ligase

14°C O/N

5 λ \rightarrow 50 λ Ag-I, plate on LB+AMP plates

picked 18 colonies L1:L2 ratio good

RLP1-18 digest w/ EcoRI



- did not get
wanted piece

- redo 343

5

22

① Nru I partial digestion of pOG336

13 λ DNA (12/5/95 prep at 1.95/ λ)
 20 λ 10X Nru I
 0.5 λ Nru I
166.5 λ H₂O
 200 λ

To 37°C, timepoints taken at 3, 6, 12, 24 and 45 minutes.

(40 λ → 4 λ 250 mM EDTA → ice)

1 λ (125 ng) of each timepoint run out on a minigel.

Plc the rest of each timepoint and leave to ppt overnight.

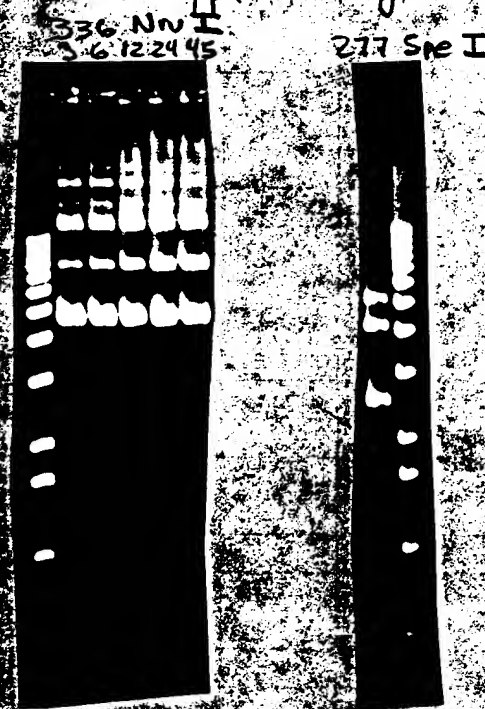
② Spe I digest of pOG277

5 λ DNA (7/30/94 prep at 1.8/ λ)
 20 λ 10X #2
 10 λ Spe I
165 λ H₂O
 200 λ

37°C for one hour

4 λ → minigel

Plc, ppt overnight



The partial turned out more incomplete than we would like - it would be better if there were less of the supercoiled and relaxed circular DNA and more of the doubly-digested DNA, but as long as we do not overload the gel and as long as we run the digest out sufficiently far, we should be OK.

The Spe I digest of 277 was not very complete - there was a substantial amount of supercoiled and relaxed circular DNA. Steve spun down the DNA, washed it, and redigested it with Spe I. He ran a small aliquot on a minigel and the cleaned up and precipitated the rest.

Redigestion of Spe I-cut 277 looks good

Spun down the Spe I-cut 277 and the 45 minute timepoint from the 336 Nru I partial. Washed with 70% EtOH, respin, and resuspended both pellets in 20 λ TE

Redigested Spe I-cut 277



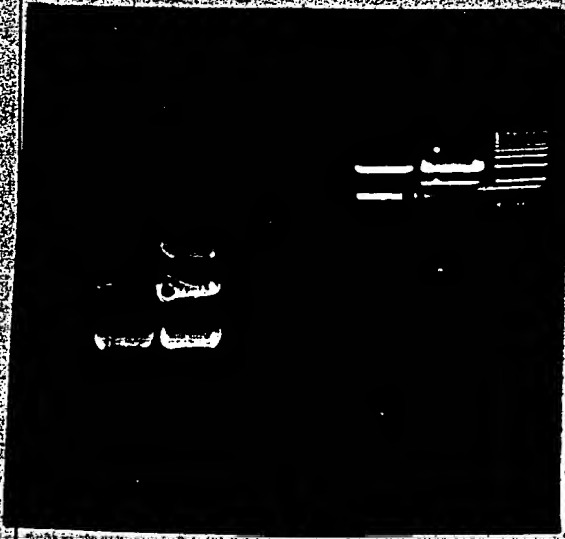
CIP 336 Nru I

20 λ DNA
5 λ 10x buffer
2 λ CIP enzyme
23 λ H₂O
50 λ

30 min. at 37°C
load 15 & 30 λ onto a 0.6% LMA/TAE gel.
Run for a long time
Want ~ 8 kb band

0.6% LMA/TAE gel

- ① kb ladder
- ② 336 Nru I (CIP) 15 λ
- ③ " 30 λ
- ④ blank
- ⑤ blank
- ⑥ 277 Spe I (klenow) Sma I 15 λ
- ⑦ " 35 λ
- ⑧ kb ladder



Klenow of 277 Spe I

20 λ DNA
2 λ 2mM dNTP's
5 λ 10x buffer
2 λ Klenow
21 λ H₂O
50 λ

30 min. at RT
70°C for 5 min.
PIC, ppt, spin, wash with 70% EtOH,
respin, resuspend in 20 λ TE

Sma I digest of 277 Spe I (Klenow)

20 λ DNA
5 λ 10x #4
5 λ Sma I
20 λ H₂O
50 λ

One hour at RT
1 λ → minigel
15 & 35 λ onto 0.6% LMA/TAE gel
(Run ~ 1 hour)
Want ~ 1200 bp band



Ligations

- ① 5 λ 336 gel
15 λ 277 gel
 - ② 5 λ 236 gel
15 λ blank gel
- BOTH:

86 λ 10mM Tris
12 λ 10x gel lig. buffer
1 λ 100mM ATP
1 λ T4 DNA Ligase

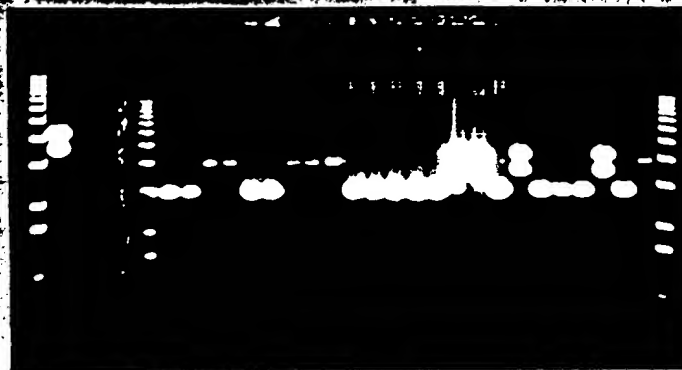
To 14°C O/N

⑤ 24

19/95 → Transformed 5 λ ligations into 50 λ Stratagene AG-1's

not many colonies on both C1, C2 plates
ratio good

picked 24 colonies for RAPDs

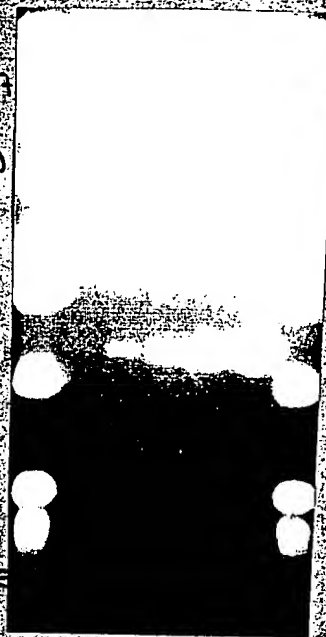


RL#1-24; 336. EURI
I do not believe that
any of these are correct.

Construction

1. BamHI partial digest of 277

0.5 λ DNA (0.87 λ 123045 QP)
 20 λ 10x #3
 1 λ BamHI
 134 λ H₂O
 33°C
 time points 3', 6', 12', 24', 45'
 1 λ of each \rightarrow minigel
 add 4x 250mM EDTA
 add 160x TE
 1x P/C, ppt w/ glycogen
 spin 70% wash
 resuspend 12' time point in 20x TE



2. Sma complete of Bam partial 277

20 λ DNA
 4 λ 10x #4
 4 λ Sma I
 12 λ H₂O

RT, 1 hr, 1 λ \rightarrow minigel, 15, 30 λ \rightarrow 1x CMA gel

3. HindIII complete of NruI partial of 336

spin down 2
 resuspend 12.96 NruI partial, 24' time point
 in 20x TE (see 336 B notes)

20 λ DNA
 4 λ 10x #2
 4 λ HindIII
 12 λ H₂O

37°C 1 hr, 1 λ \rightarrow minigel
 15, 30 λ \rightarrow 0.61 CMA TAE gel

4. BglII + HindIII digest of 336B

25 λ 336B RCM1
 5 λ 10x #2
 2.5 λ BglII
 2.5 λ HindIII
 1.5 λ H₂O

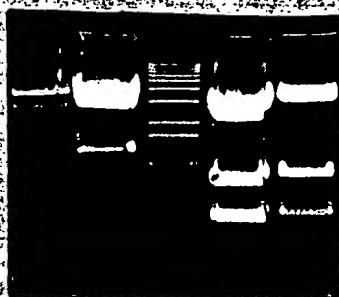
37°C 1 hr, 1 λ \rightarrow minigel
 15, 30 λ \rightarrow 1x CMA gel



277 Sma + Bam(P)
 336 HindIII + Nru(P)



1% LMA gel



336B

277 Bam(P) + Sma

336 Nru(P) + Hind III



336B Bgl II + Hind III

ligations

L1: 3 λ 336 Nru(P) + Hind III gel
 S λ 277 Bam(P) + Sma I gel
 S λ 336B Bgl II + Hind III gel

L2: =
 10 λ 1.0% blank

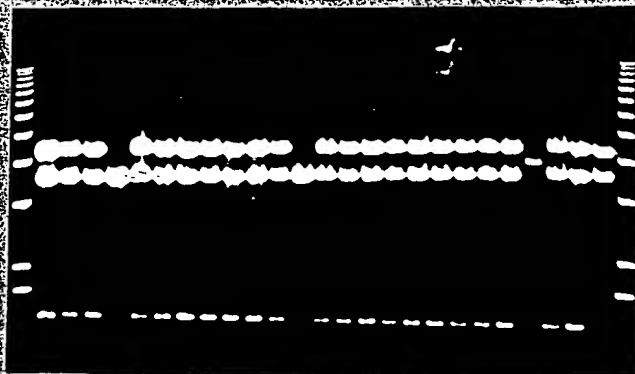
both 12 λ 10x gel
 1 λ 100mM ATP
 1 λ T4 ligase
 9 λ 10mM Tris (pH 7.4)

140C 10W

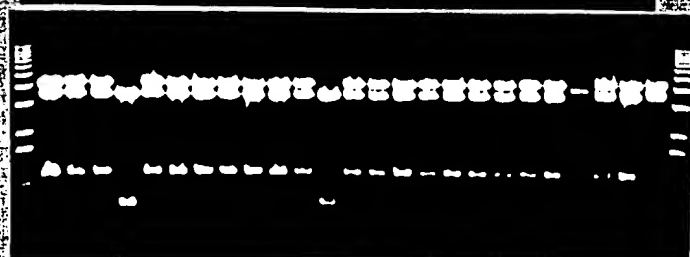
5 λ 502 Ag-I plated w/ Amp

Picked 24 colonies for rapid's. L1, L2 ratio not good
 a low number of colonies on both plates

343 R1+11=24 control 336 w/ EcoRI



R1+1-3, 5-11, 13-21, 23, 24 looks correct



6/28

343 RL #1

Bam HI

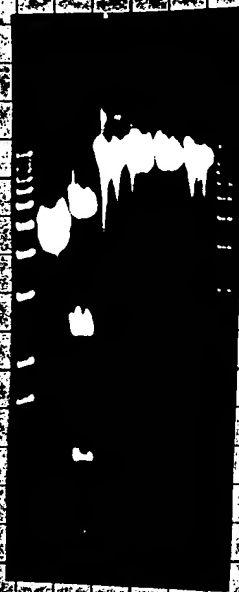
Sac I

Not I

Sal I

Kpn I

Hind III



Standards

<u>Conc.</u>	<u>Read #1</u>	<u>Read #2</u>
0	0	12
100	104	109
200	193	201
300	312	302
500	482	480
750	746	729
1000	1000	968

Samples. 3 λ sample into 3ml assay buffer.

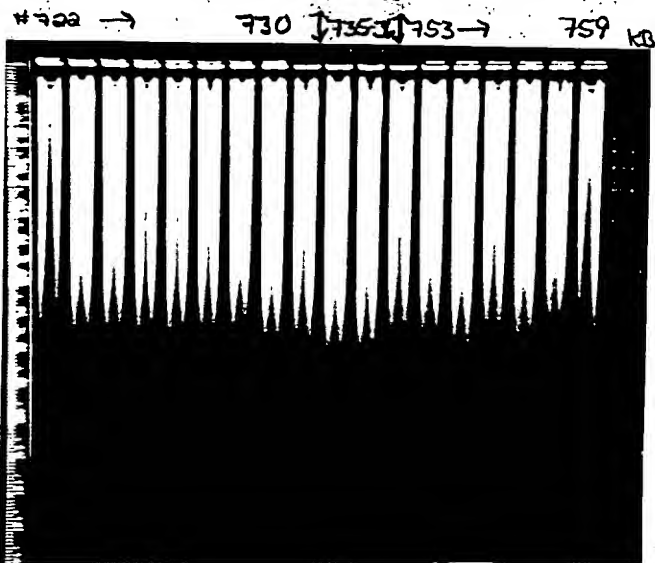
<u>#</u>	<u>Conc. (ng/λ)</u>	<u>λ / 10 μg</u>	<u>10x (6λ)</u>	<u>Enzyme (6λ)</u>	<u>H₂O</u>
722	342	29 λ	BamHI	BamHI	19 λ
723	360	28			20 λ
724	444	23			25
725	397	25			23
726	431	23			25
727	295	34			14
728	450	22			26
729	378	27			21
730	440	23			25
735	791	15	*3	PstI	33
736	694	15			33
753	294	34	*3	PstI	14
754	540	19			29
755	415	24			24
756	520	19			29
757	317	32			16
758	547	18			30
759	434	23			25
722		29	*3	PstI	19
723		28			20
724		23			25
725		25			23
726		23			25
727		34			14
728		22			26
729		27			21
730		23			25

1:40 pm

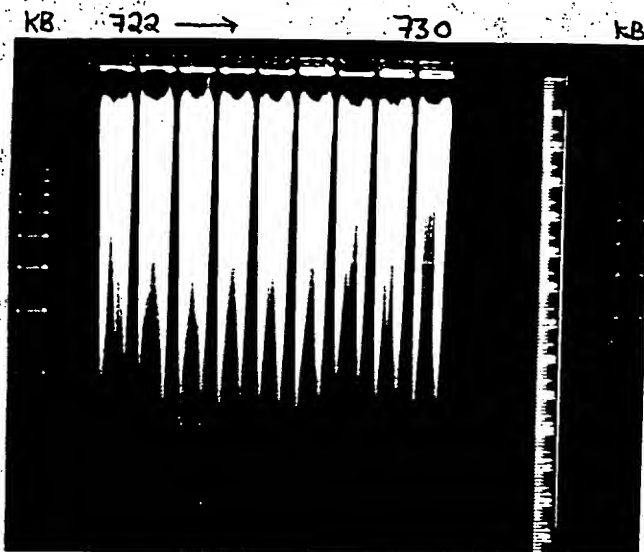
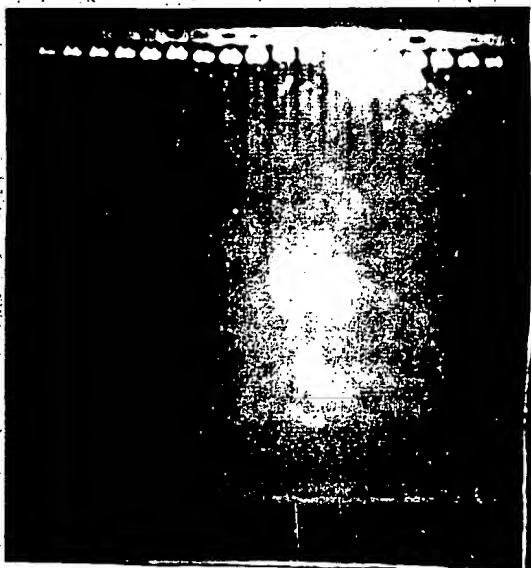
(90) / 30

2, 7, 9, 10, 11

SOUTHERN HYBRIDIZATION OF P2-S/304 M MOOSE TAIL DNA's



Pst I digests 12/69SA
P2-S P2-1 probe 12-18-95



BamHI digests 12/69SB
304M Cre probe



[illegible]

121695A

Pa-5

P2-1 probe

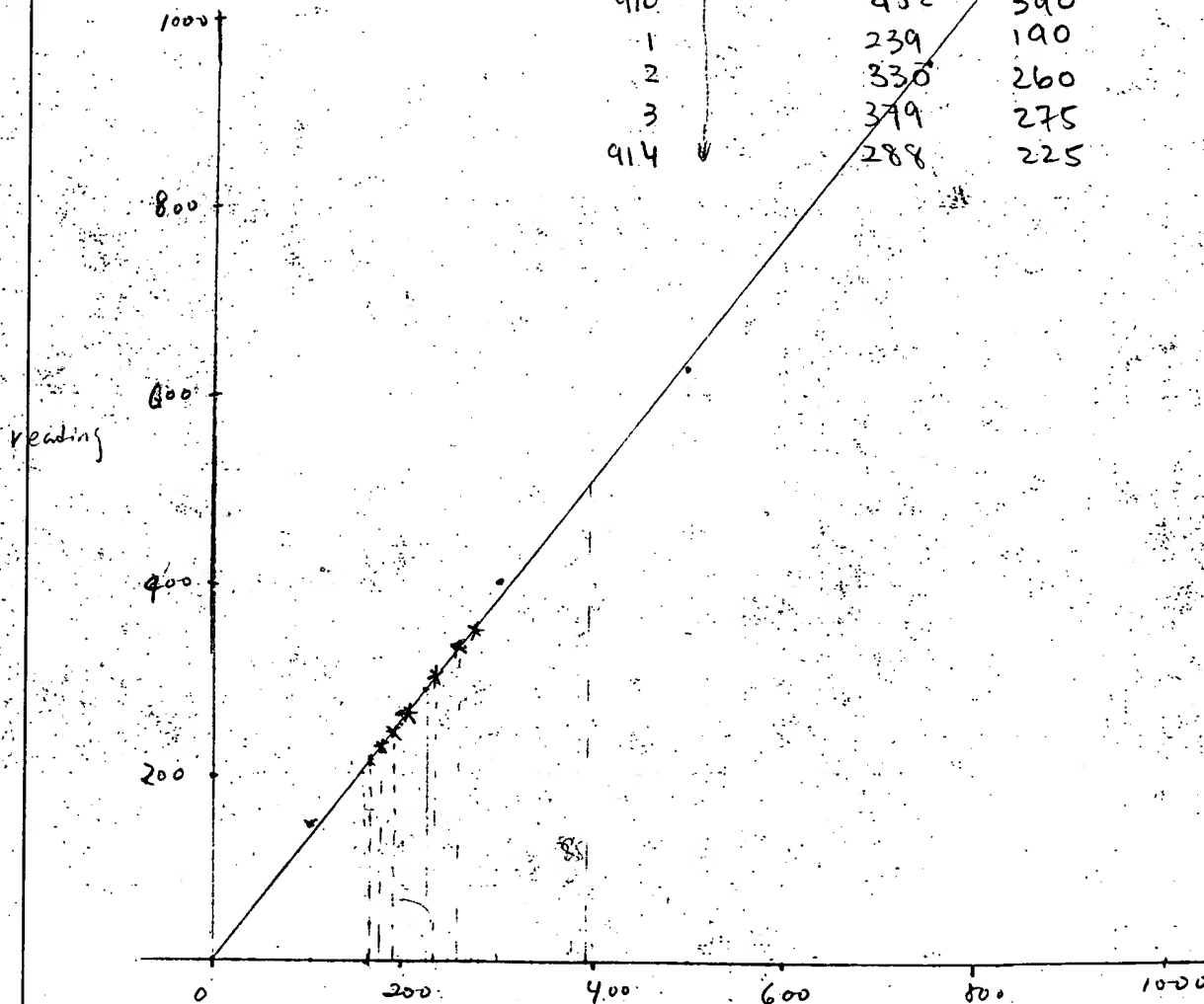
24 hrs. exposure

Mouse DNA Quantification

Standard	reading
0	0
100	145
200	267
300	401
500	632
750	957
1000	1017

Sample #	reading	acc. ng/μl
894*	346	280
895	225	175
6	264	200
7 (6λ)	329	260
8	242	190
9	298	230
900	261	200
1	243	190
2	248	190
903 (6λ)	211	160
4 (9λ)	287	225
5	298	230
6	253	195
7	295	230
8 (18λ)	32	/
9 (9λ)	203	180
910	452	390
1	239	190
2	330	260
3	379	275
914	288	225

mouse # 894* - 914
Embryo.

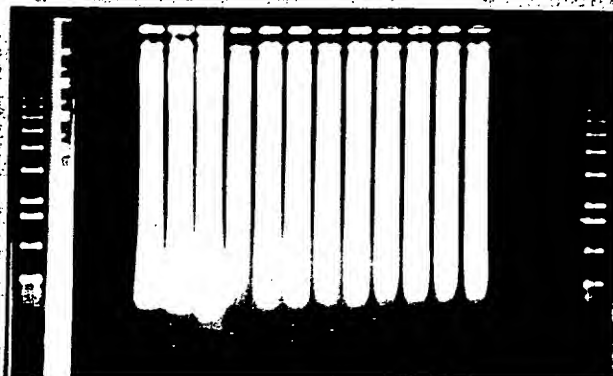


mouse Embryo DNA: genotyping digest w/ PstI

lane #		DNA conc. ng/λ	DNA λ	H ₂ O λ	10x #3 λ	Eng
1	1 kb					
2-4	-					
5	TL	500	20	38	7λ	5λ
6	121495	600	17	41	↓	↓
7	102595 #5	212	47	17		
8	894*	280	36	22		
9	895	175	57	3		
10	896 ✓	200	50	8		
11	897 ✓	260	38	20		
12	898 ✓	190	54	14		
13	899	230	44	14		
14	900	200	50	8		
15	901	190	54	14		
16	902 ✓	190	54	4		
17-19	-					
20	1 kb					

22 V 0.1N

Standard transfer, to prehyb for 8 hrs

P2-1 probe reading ~ 3×10^5
used all ~ 100λ

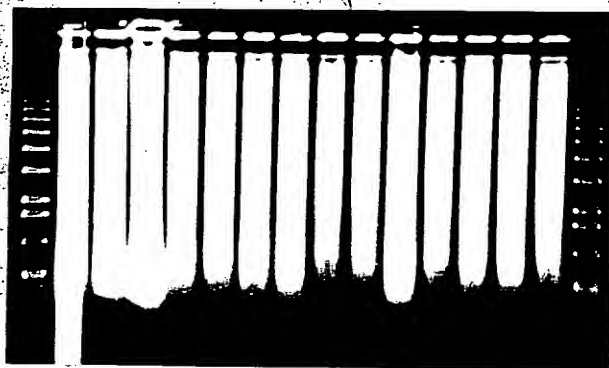
gel c

mouse Embryo DNA genotyping. Digest w/ Pst I

lane #		DNA conc ng/λ	DNA λ	H ₂ O	10x #3	Enz.
1	1Kb					
2	TL	500	20	74	11λ	5λ
3	121495	600	17	77		
4	102595 #5	212	47	47		
5	903		90	Ø		
6	904					
7	905					
8	906					
9	907					
10	909					
11	910					
12	911					
13	912					
14	913					
15	914					
16	1Kb					

22 V 01N

Standard transfer to prehyb for 8 hrs

P2-1 probe reading $\approx 2 \times 10^5$
used all (n100λ)

Blots of Embryos 894 to 914

- Today Nicky developed 20 hour exposures of blots 010696 A and B after these had been probed with a Cre probe . The signal was very strong and the A blot was very clean. The 910 sample of the B blot had an enormous signal in the 910 lane, which was the suspicious lane in the last probing with β -GAL. It would appear to me that somehow the sample that was loaded into this lane was contaminated with a Cre plasmid.
- The blot establishes that not all embryos showing the P2Br allele also contain the procre gene.
- The blots were discarded

Embryo #	Pol II	Pro Cre	β -GAL Stain
894	+ / +	+ / +	NEG
895	+ / +	+ / +	NEG
896	+ / P2Br	+ / +	POS
897	+ / P2Br	+ / 304	POS
898	+ / P2Br	+ / +	POS
899	+ / +	+ / 304	NEG
900	+ / +	+ / +	NEG
901	+ / +	+ / +	NEG
902	+ / P2Br	+ / 304	POS
903	+ / P2Br	+ / +	POS
904	+ / +	+ / 304	NEG
905	+ / P2Br	+ / +	POS
906	+ / P2Br	+ / +	POS
907	+ / P2Br	+ / +	POS
908	?	?	POS
909	+ / P2Br	?	POS
910	+ / P2Br	?	POS
911	+ / P2Br	+ / +	POS
912	+ / P2Br	+ / 304	POS
913	+ / +	+ / 304	NEG
914	+ / +	+ / 304	NEG
TOTAL			

22 121494 02576 694 695 696 697 698 699 900 901 902

72 124 125 203 204 205 206 207 208 209 210 211 212 213 214

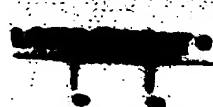
11 11 11 11 11 11 11 11 11 11 11 11 11 11 11



53
h6h121
h6S201
h694
h695
h696
h697
h698
h699
h600
h601

24 hr. ap

915 914 913 912 911 910 909 908 907 906 905 904 903 902 901 900 899 898 897 896 895 894 893 892 891 890 889 888 887 886 885 884 883 882 881 880 879 878 877 876 875 874 873 872 871 870 869 868 867 866 865 864 863 862 861 860 859 858 857 856 855 854 853 852 851 850 849 848 847 846 845 844 843 842 841 840 839 838 837 836 835 834 833 832 831 830 829 828 827 826 825 824 823 822 821 820 819 818 817 816 815 814 813 812 811 810 809 808 807 806 805 804 803 802 801 800 799 798 797 796 795 794 793 792 791 790 789 788 787 786 785 784 783 782 781 780 779 778 777 776 775 774 773 772 771 770 769 768 767 766 765 764 763 762 761 760 759 758 757 756 755 754 753 752 751 750 749 748 747 746 745 744 743 742 741 740 739 738 737 736 735 734 733 732 731 730 729 728 727 726 725 724 723 722 721 720 719 718 717 716 715 714 713 712 711 710 709 708 707 706 705 704 703 702 701 700 699 698 697 696 695 694 693 692 691 690 689 688 687 686 685 684 683 682 681 680 679 678 677 676 675 674 673 672 671 670 669 668 667 666 665 664 663 662 661 660 659 658 657 656 655 654 653 652 651 650 649 648 647 646 645 644 643 642 641 640 639 638 637 636 635 634 633 632 631 630 629 628 627 626 625 624 623 622 621 620 619 618 617 616 615 614 613 612 611 610 609 608 607 606 605 604 603 602 601 600 599 598 597 596 595 594 593 592 591 590 589 588 587 586 585 584 583 582 581 580 579 578 577 576 575 574 573 572 571 570 569 568 567 566 565 564 563 562 561 560 559 558 557 556 555 554 553 552 551 550 549 548 547 546 545 544 543 542 541 540 539 538 537 536 535 534 533 532 531 530 529 528 527 526 525 524 523 522 521 520 519 518 517 516 515 514 513 512 511 510 509 508 507 506 505 504 503 502 501 500 499 498 497 496 495 494 493 492 491 490 489 488 487 486 485 484 483 482 481 480 479 478 477 476 475 474 473 472 471 470 469 468 467 466 465 464 463 462 461 460 459 458 457 456 455 454 453 452 451 450 449 448 447 446 445 444 443 442 441 440 439 438 437 436 435 434 433 432 431 430 429 428 427 426 425 424 423 422 421 420 419 418 417 416 415 414 413 412 411 410 409 408 407 406 405 404 403 402 401 400 399 398 397 396 395 394 393 392 391 390 389 388 387 386 385 384 383 382 381 380 379 378 377 376 375 374 373 372 371 370 369 368 367 366 365 364 363 362 361 360 359 358 357 356 355 354 353 352 351 350 349 348 347 346 345 344 343 342 341 340 339 338 337 336 335 334 333 332 331 330 329 328 327 326 325 324 323 322 321 320 319 318 317 316 315 314 313 312 311 310 309 308 307 306 305 304 303 302 301 300 299 298 297 296 295 294 293 292 291 290 289 288 287 286 285 284 283 282 281 280 279 278 277 276 275 274 273 272 271 270 269 268 267 266 265 264 263 262 261 260 259 258 257 256 255 254 253 252 251 250 249 248 247 246 245 244 243 242 241 240 239 238 237 236 235 234 233 232 231 230 229 228 227 226 225 224 223 222 221 220 219 218 217 216 215 214 213 212 211 210 209 208 207 206 205 204 203 202 201 200 199 198 197 196 195 194 193 192 191 190 189 188 187 186 185 184 183 182 181 180 179 178 177 176 175 174 173 172 171 170 169 168 167 166 165 164 163 162 161 160 159 158 157 156 155 154 153 152 151 150 149 148 147 146 145 144 143 142 141 140 139 138 137 136 135 134 133 132 131 130 129 128 127 126 125 124 123 122 121 120 119 118 117 116 115 114 113 112 111 110 109 108 107 106 105 104 103 102 101 100 99 98 97 96 95 94 93 92 91 90 89 88 87 86 85 84 83 82 81 80 79 78 77 76 75 74 73 72 71 70 69 68 67 66 65 64 63 62 61 60 59 58 57 56 55 54 53 52 51 50 49 48 47 46 45 44 43 42 41 40 39 38 37 36 35 34 33 32 31 30 29 28 27 26 25 24 23 22 21 20 19 18 17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1 0



Mouse Embryo Genotyping-Southern Blots

Nicky developed the films of Min's blots that had been up to hyb since yesterday. These blots (010696 A and 010696B) contained samples of genomic DNA from embryos that were stained for β -GAL activity and which have been scored previously, see above. The blots were reasonably clean and the signal was strong.

Blot 010696A contained samples from embryos #'s 894* to 902*

- this blot contained the above samples and control DNA's from normal es cells, the original recombinant, and the Cre recombinant.
- None of the mouse samples showed the band expected for the original recombinant, they either showed only the wt allele or the wt allele and one copy of the Cre recombinant allele. The four animals showing the Cre recombinant allele were those that had been scored as β -GAL positive previously (#896, 897, 898, and 902).

Blot 010696B contained samples from embryos #'s 903 to 914 with the exception of 908, a sample that was lost during DNA extraction.

- 8 of 11 samples on the blot showed the pattern of hybridization anticipated for the Cre recombined allele (903, 905, 6, 7, 9, 910, 911, 912). Three animals only showed the pattern associated with the wt allele (904, 913, 914).
- all of the animals that I had previously scored as β -GAL positive showed the Cre recombined band on this blot.
- Sample 910 poses a real problem. In addition to the wt band and the Cre recombined band this animal shows a band of the same size as the original recombinant and some additional apparently poorly digested DNA. The wt and p2br bands are present in approximately equimolar amounts, as would be expected, and they are of approximately the same intensity as the other samples. The additional p2bc band is stronger than either of these two, and would therefore have to represent an amplification of the unrecombined allele if it were to be real. It may well be that the expression of Cre in spermatids leads to some unusual rearrangements in addition to the anticipated rearrangements. Before getting too excited about this prospect it would be useful to extract DNA from the β -GAL-stained carcass if possible and to determine if this DNA shows the same pattern.

Friday,

Scoring embryos 903-914

- 903: I score this embryo as clearly positive, although the uniformity of staining is clearly disappointing. The most intense staining is in the GI tract and mouth structures, but there is clear staining of the somites as well. The vibrissal follicles are positive and the choroid plexus is also positive.
- 904: This embryo is entirely negative.
- 905: I score this animal positive with some reservations. The embryo was bisected coronally at the level of the forelimb. One side of the brain, viewed through the skin, is clearly positive. The other does not appear to be so. Some of the GI tract looks to be clearly positive, and the vibrissal follicles are faintly positive.
- 906: I score this embryo as positive in much the same sense as for 903. Some parts of the nervous system are reasonably well stained.
- 907: This embryo is very clearly positive. The CNS is well-stained as are the somites. Even in this embryo there is very considerable variation in the intensity of staining from region to region.
- 908: This embryo is positive in the 903 sense. The CNS is well, but variably, stained. The vibrissal barrels are faintly stained.
- 909: This embryo is clearly positive. The staining is intense in many places but it is blotchy.
- 910: This embryo is positive in the 903 sense.
- 911: This embryo is positive in the 903 sense.
- 912: This embryo is positive in the 903 sense, with some striking vibrissal staining and good forebrain staining.
- 913: This animal is clearly negative.
- 914: This animal is clearly negative.

894-902 Positives = 896, 897, 898, and 906

Friday,

Blots of Embryos 894 to 914

• Today Nicky developed 20 hour exposures of blots 010696 A and B after these had been probed with a Cre probe. The signal was very strong and the A blot was very clean. The 910 sample of the B blot had an enormous signal. This lane gave a puzzling result in the first probing of this blot with β -GAL; there were signals consistent with the other lanes that indicated that there was one wild-type allele and one P2Br allele, but there was additional signal. One part of this additional signal ran at about the same level as the P2Bc allele, another part of this signal was fairly high mw DNA. In the cre probing the whole lane is a smear - the kind of signal one sees in a blot of plasmid DNA when hundreds of ng of DNA are loaded. The only way to determine what is going on in this lane will be to run another gel with the newly extracted DNA from the rest of the embryo.

- The blot establishes that not all embryos showing the P2Br allele also contain the ProCre allele.
- The blots were discarded

• Overall, there were 21 embryos, 12 of the 19 that could be genotyped for RNA-PolIII had the P2Br allele (none had p2bc), 7 of 18 that could be genotyped for ProCre were positive, and 13 of 21 were positive for β -GAL. The correlation between β -GAL staining and the P2Br allele is 1:1.

Embryo #	Pol II	Pro Cre	β -GAL Stain
894	+/+	+/+	NEG
895	+/+	+/+	NEG
896	+/P2Br	+/+	POS
897	+/P2Br	+ / 304	POS
898	+/P2Br	+/+	POS
899	+/+	+ / 304	NEG
900	+/+	+/+	NEG
901	+/+	+/+	NEG
902	+/P2Br	+ / 304	POS
903	+/P2Br	+/+	POS
904	+/+	+ / 304	NEG
905	+/P2Br	+/+	POS
906	+/P2Br	+/+	POS
907	+/P2Br	+/+	POS
908	?	?	POS
909	+/P2Br	?	POS
910	+/P2Br	?	POS
911	+/P2Br	+/+	POS
912	+/P2Br	+ / 304	POS
913	+/+	+ / 304	NEG
914	+/+	+ / 304	NEG

New Blot of Embryos 894-902

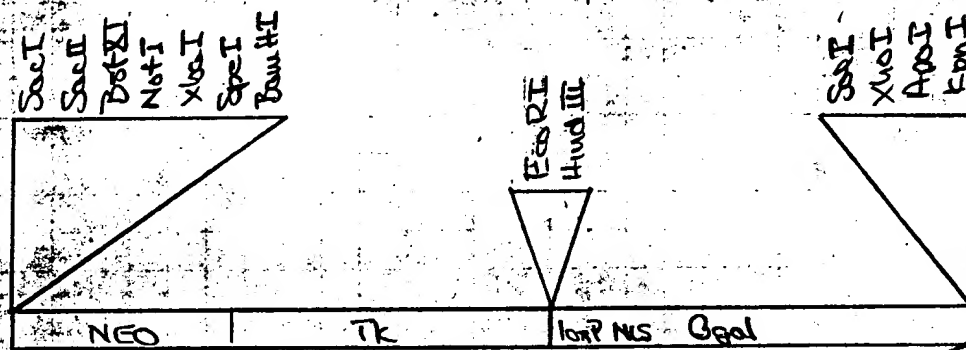
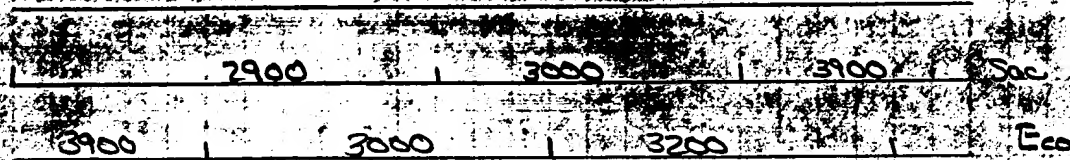
- The first Southern blot (010696A) of these embryos looked pretty good - the samples are appropriate for attempting to make a publication Southern. Nicky poured a new 0.6% gel and did Pst I digests of the DNA's that were on that blot, and did an additional digest of 392 DNA. The expectation is that the somatic (tail)DNA of the 392 mouse, which sired this litter, will show the P2Bc allele, while the embryos he sired will show the P2Br allele. We did not have hoe assay results for the samples of 392 that we had previously prepared so Nicky read the OD 260 and we estimated that it would be high by about 1/3, so instead of running a nominal 10 γ of DNA we ran a nominal 15 γ of DNA - the latter should come close to a true 10 γ of DNA because the sample is heavily contaminated with RNA.
- The gel was run overnight at 24 volts to minimize band smiling. On Wednesday it will be transferred and put up to prehyb overnight - it will be probed with P2-1 overnight on Thursday

Neo^R + TK^R + loxP + Bgl

MUTANT NEO

235

235^c Is Corrected Version



Vector = pOG227 cut with HindIII and SalI

Insert = pOG230 cut with HindIII and SalI

6/45

Digests for 235^c and 239^c

235
239

① Hind III digest of 227^c RL #1

25 λ DNA
20 λ 10x #2
10 λ Hind III
145 λ H₂O

② Hind III digest of 235^c

20 λ DNA 1019/30 268 λ
20 λ 10x #2
10 λ Hind III
168 λ H₂O

③ Hind III digest of 239^c

1.5 λ DNA 1212/30 3.35 λ
20 λ 10x #2
10 λ Hind III
168 λ H₂O

5 λ each digest \rightarrow minigel
balance cleared up and noted

1/11/94

DNA spun down, resuspended in 20 λ , 1 λ into minigel.

Bam HI digests

Minigel

10 λ DNA
5 λ 10x Bam
5 λ Bam HI
20 λ H₂O

1/20th 227^c Hind
1/10th 227^c + Bam
1/20th 235 Hind
1/20th 235 + Bam
1/20th 239 Hind III
1/10 239 + Bam

Realized that I needed another strategy for 239^c

\therefore Kpn I digest 239 cut with Hind III

10 λ DNA
5 λ 10x #1
5 λ Kpn I
20 λ H₂O

45 minutes at 37 $^{\circ}$ C, then 2 λ \rightarrow minigel

25 λ reserved on ice
25 λ for Bam HI digest
+5 λ 100 mM Tris
+5 λ 100 mM NaCl
2.5 λ 100 mM MgCl₂
+2.5 λ Bam HI
+1.5 λ H₂O

6/46

150 x 10 =
1500

5M
1.5 = 3.3 x

① Digest pOG230 with Hind III

have 201 DNA from Giagen prep of 230, from colony #4
OD 260/280 done on 1:100 dilution

SAMPLE	A320	A280	A260	280/260	260/280
1.0000	0.0070	0.3048	0.5816	0.5182	1.9296

Concentration of DNA = 2.98/1. Will be isolating 3kb fragment from 6kb plasmid. Digest about 50 DNA

21 DNA
201 10X #2
101 Hind III
1681 H₂O
31 → minigel.
clean and ppt balance

② Digest pOG227 with Hind III

have Giagen prep from 1012/93 = 1.68/1

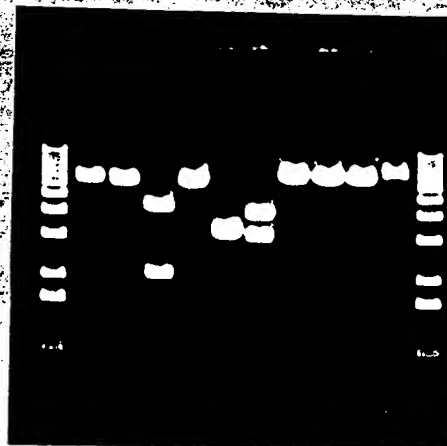
31 DNA
201 10X
101 Hind III
1671 H₂O
41 → minigel
clean and ppt balance



Check digests on plasmids, 100 ug each

#230
Epn I (1)
Hind III (3)
Sac I (1)
Sal I (5)
Eco RI (5)

#227
Eco RI
Bam HI
Hind III
Sal I
Sma I (1)



6/47

DNA's spun down, washed, resuspended in 40 μ TE

Sal I digest (2 $^\circ$) 230

Sal I digest (2 $^\circ$) 227

DNA 20 μ
 10x 5 μ
 SRI 5 μ
 H₂O 20 μ

DNA 20 μ
 10x 5 μ
 SRI 5 μ
 H₂O 20 μ

LMA gel: 0.6% LMA in TAE



The two fragments of 230 were not as separated as I would have liked.

Ligations

#1 15 μ 230 gel
 5 μ 227 gel

#2 15 μ blank gel
 5 μ 227 gel

5 μ of each gel ligation was used to transform 50 μ aliquots of AG-P. Standard plating. 12/15/93

8 colonies picked into 5 μ LB + Amp. (plates gave very good transformation ratio. 10/17/93

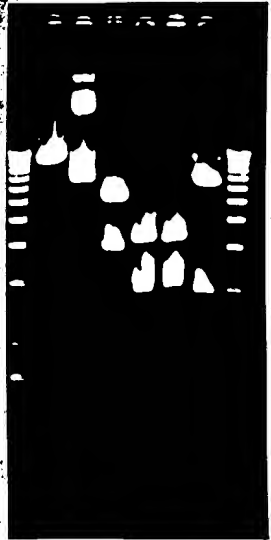
Std. repeats done. Picked up in 50 μ . 1 μ digested with EcoRI 12/18/93



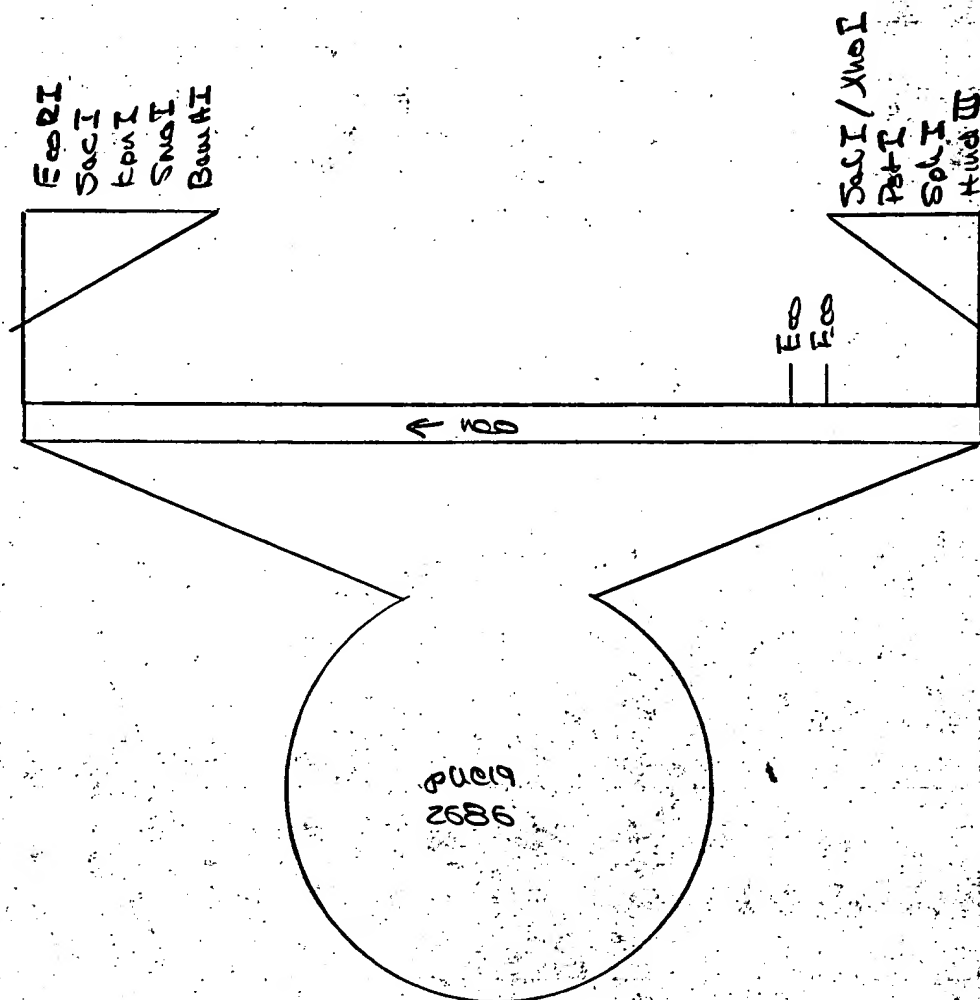
Overloaded. 1, 2, 3, 4, 7 look to be correct.

#1 & 2 seeded for Qiagen preps

Additional checks on 235 RC #1.



- 1) Hind III
- 2) Xba I
- 3) Hind III + Xba I
- 4) Sac I
- 5) Eco RI
- 6) Sal I + Bam HI



① Digest pMCI neo with BamHI 5/28/90 prep is 5.78/λ

1λ DNA
20λ 10x Bam
10λ BamHI
169λ H₂O



1. 229
2. pUC
3. pMCI-1

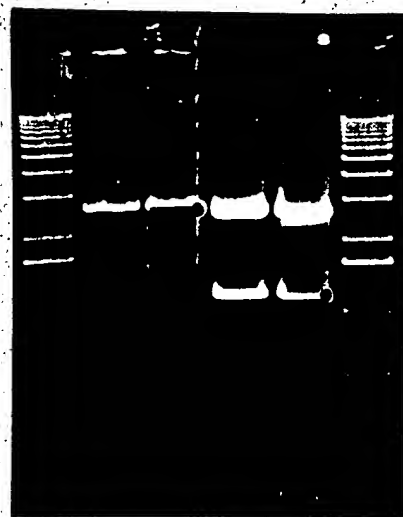
② Digest pUC19 with BamHI (TP prep at 0.98/λ)

5λ DNA
20λ 10x Bam
10λ Bam
169λ H₂O

Both digests run one hour at 37°
Extracted and potted overnight.
Resuspended in 25λ TE
1λ → minigel.

③ Digest Bam - MCI neo with XbaI

15λ DNA
5λ 10x X
5λ XbaI
25λ H₂O



④ Digest Bam - pUC with SmaI

15λ DNA
5λ 10x Sma
5λ SmaI
25λ H₂O

LMA gel: pUC19 15λ
35λ
MCI neo 15λ
35λ

Bands excised and left in refig overnight.

12/18 Standard ligations set up

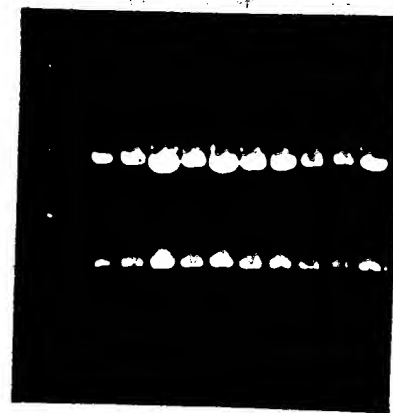
12/19 Transformed into AG-1

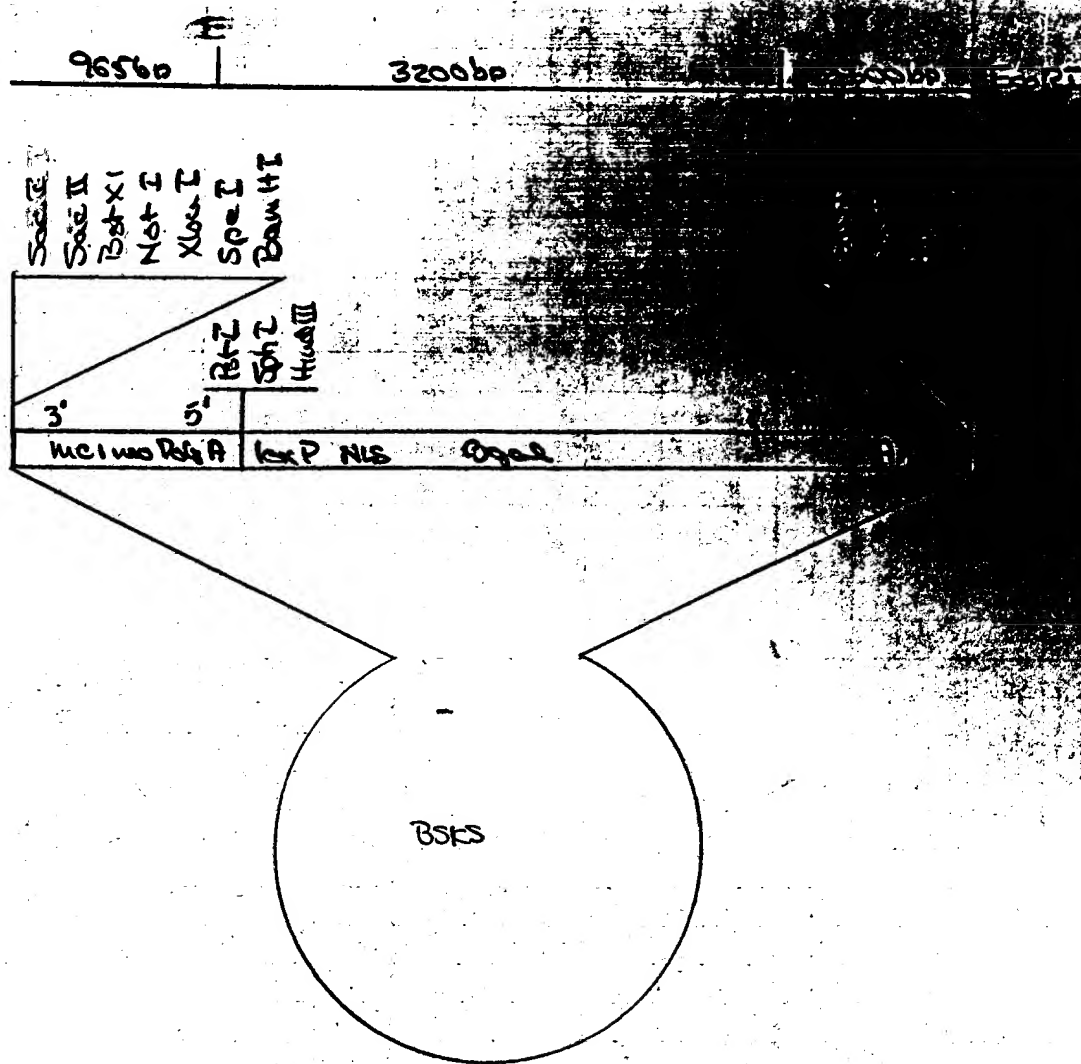
12/20 Good transformation ratio - 10 colonies picked.

6/51

Repeats digested with EcoRI.

all colonies look to be correct
#1 & #2 saved.





McIneo Pgly A cassette from pCG 247 (known to be wild-type)
Bgal and vector sequences from pCG 235

This plasmid is part of a replacement vector for the Pbl II locus.

6/53

① Digest pOS247 with HincIII

21 DNA 12/27/93 prep at 358/1
 201 10x#2
 101 HincIII
 1681 H₂O

② Digest pOS235 with HincIII

21 DNA (10/19/93 prep at 268/1)
 201 10x#2
 101 HincIII
 1681 H₂O

41 each digest loaded onto minigel
 DNA cleaned up and precipitated
 resuspended in 201 TE

③ BamHI 2' digest 247 and 235

201 DNA
 51 BamHI
 51 Bam
 201 H₂O

digested one hour
 21 -> minigel

201 of 235 digest and 351 of 247 digest loaded onto minigel

Bands excised and standard ligations set up.

L1 51 235 gel
 151 247 gel
 121 10x gel lig buff
 11 100 uM ATP
 11 T4 ligase
 861 10 uM Tris

L2 =
 151 blank
 =
 =
 =
 =

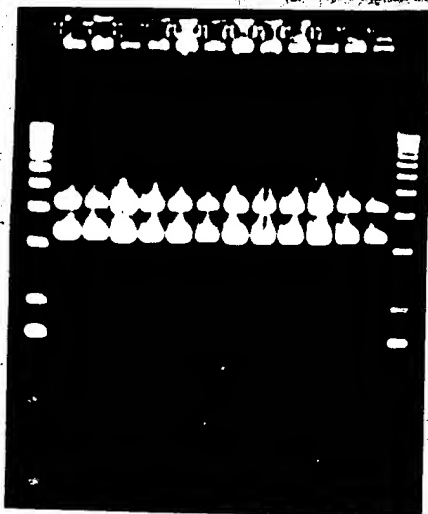
1/5/93 51 aliquots of ligations were used to transform 501 aliquots of AG-1.

1/6/93 Excellent transformation ratio obtained 710:1

6/54

Standard boiling rapid lysates. Up in 100%

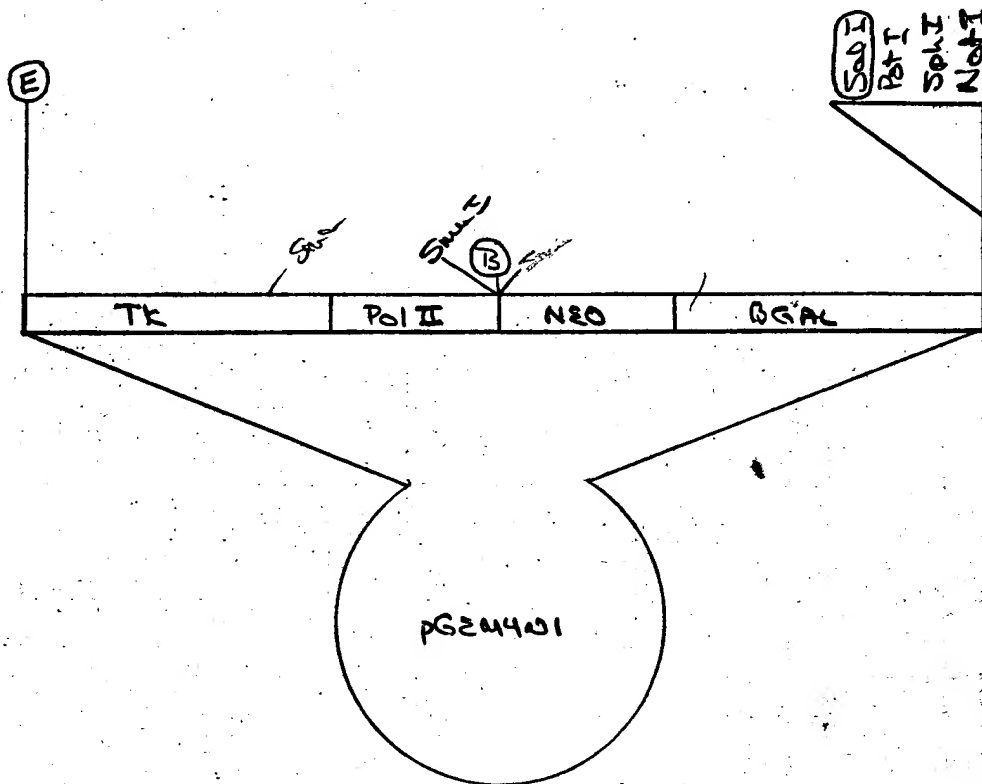
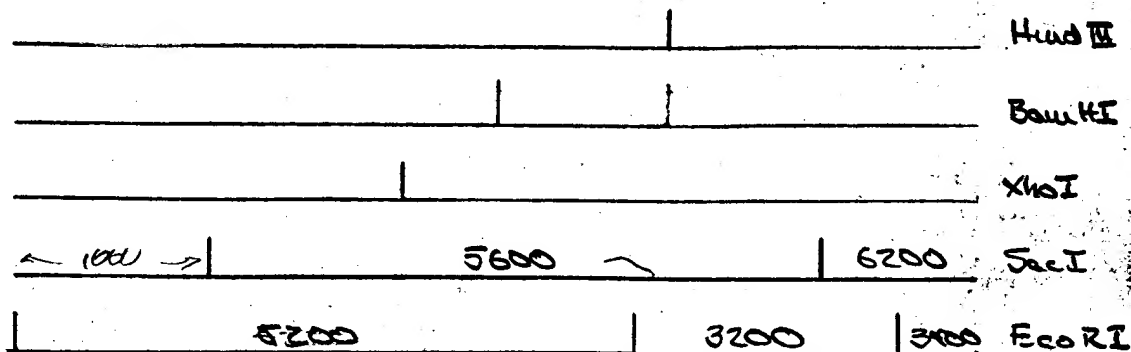
1.1 aliquots digested with EcoRI



All samples yield fragments predicted for correct recombinant.

TK+Pol II plus Neo + Bgal for Replacement.

25



EcoRI to BamHI (4800bp) from 245 T.

BamHI to SalI (4800bp) from 250

SalI + EcoRI (2900bp) from pGEM401

1/56

Replacement

① EcoRI + BamHI digest 245

10 λ 245 RL #1
5 λ 10X #3
2.5 λ EcoRI
2.5 λ BamHI
30 λ H₂O

Minigel 5 λ each digest loaded on minigel.



② BamHI + SalI digest 250

10 λ 250 RL #1
5 λ 10X Sal
3 λ SalI
2 λ BamHI
30 λ H₂O

③ Eco + Sal digest pGEM4NI

1 λ DMSO
5 λ 10X Sal
3 λ SalI
2 λ EcoRI
38 λ H₂O

1/22/93 CMA gel 30 λ of 245, 250 digests and 10 and 20 λ of pGEM digest was loaded onto 0.6% CMA gel

Loaded Gel Incorrectly

Redo digests as above 5 λ each digest \rightarrow minigel

20 λ pGEM, 30 λ 245, 40 λ 250 loaded on CMA gel.

Ligations:

L1 5 λ pGEM L2 5 λ pGEM
7.5 λ 245 15 λ blank
7.5 λ 250

Other components standard.

1/23 10 λ each ligation \rightarrow 100 λ AG-1

1/24 excellent ratio obtained
12 colonies \rightarrow AG-1



6/57

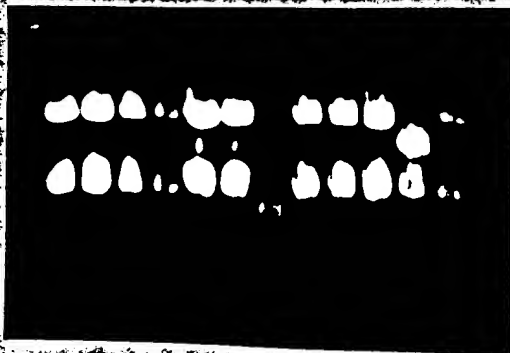
Std boiling controls, EcoRI digests:



All but 7 and 11 look correct

~3000bp doublet clearest in
#2

#2 and #6 saved.

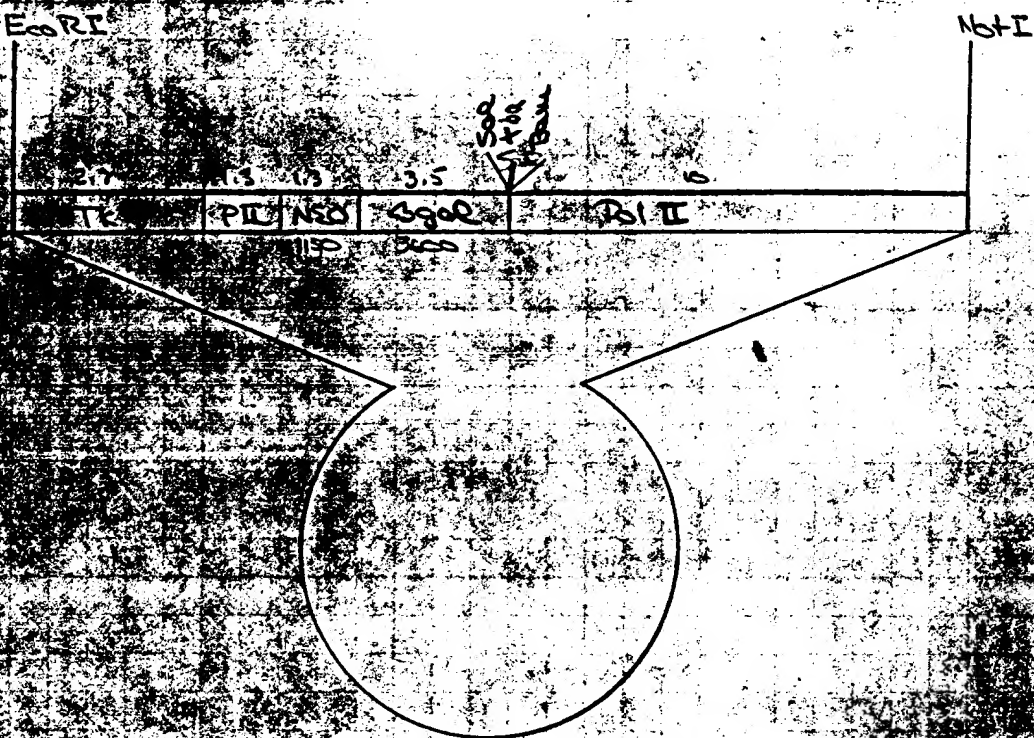
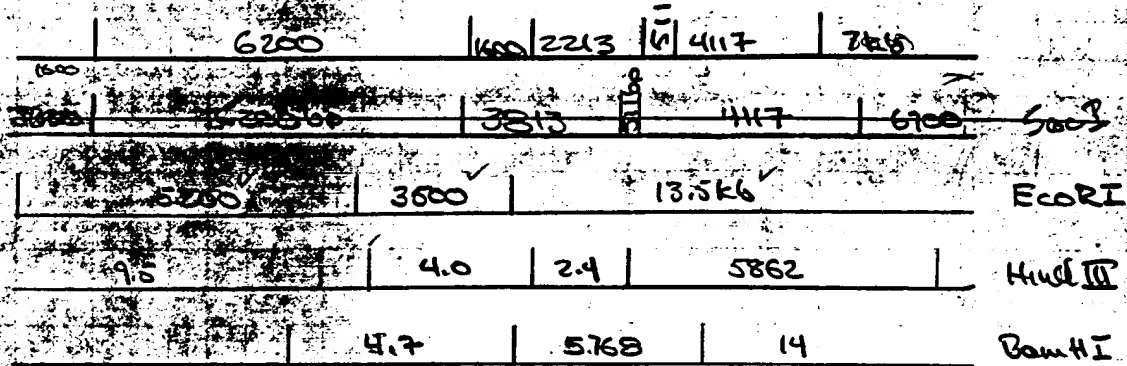


Numerous additional digests done for construction of 260-sec notes

#2 saved for Guyen prep 1/26

Poll II - Bgal Replacement Vector

260



Vector = 254 cut with Sal I and Not I. Includes T₇ promoter, ori, Bgal, (ca 11.4kb)

Insert = 255N cut with Sal I and Not I. Includes ca. 11.4kb of Pst II genomic sequence

6/59

Further checks on 254 RL #2

- | | |
|-------------|-------|
| 2 EcoRI | (Eco) |
| 4 BamHI | (Bam) |
| 1 XhoI | (#2) |
| 3 Eco + Bam | (#3) |
| 5 NotI | (#3) |
| 6 SalI | (Sal) |
| 7 Sal + Bam | (Sal) |



Preparative digest 254

- 10λ 254 RL #2
- 5λ 10λ Sal
- 3λ SalI
- 2λ NotI
- 30λ H₂O

Preparative digest 255N

- 1λ 255N prep 1/14/94

- ① Add all components except NotI
- ② remove 5λ → 37°
- ③ Add NotI

Urea gel

- 1) 15λ 254
- 2) 35λ 254
- 3) 40λ 255N
- 4) 10λ 255N



- 1/10 254 Not + Sal
- 1/10 " Sal only
- 1/10 255N Not + Sal
- 1/10 255N Sal only

It appears that the SalI digest of 254 may be incomplete.

Urea gel - want to use about 2x the amount of 255N to get good concentration of fragments

New Digests

20 μ 254 RE #2 (now diluted & compared to previous digest)

20 μ 10x SCL

15 μ SCL

155 μ H₂O

1, 3, 6 μ of each digest

3 μ 255 N prep 1/19/94

20 μ 10x SCL

10 μ SCL

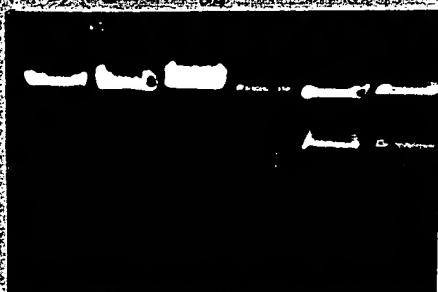
167 μ H₂O

digests cleaned up and noted overnight.
resuspended in 20 μ TE.

1/27/94

- 2, 6, and 10 μ aliquots of 1^o digest were
redigested with Not I in V_r = 40 μ , with
4 μ enzyme and

- after one hour digestion, each digest was
loaded into a 0.6% TAE CMA gel.
run at 35V



Indicated bands excised (photo in
next section)

Ligations

L1 3 μ 254 gel
7 μ 255 N gel

L2 3 μ 254
7 μ 255 N

1/28/94

10 μ each ligation was used to
transform 100 μ aliquots of PG1

+ 6 μ 10x
+ 1 μ ATP
+ 1 μ ligase
+ 40 μ 10 mM Tris

1/29/94

Obtained approximately 10:1 L1:L2 ratio

1/30/94

12 colonies picked into LB + Amp.

1/31/94

800 colonies, digested with Hind III

all look correct.

#1 & 2 saved

#1 restreaked, seeded.



36 ♂ ? Southern not clear sac

p2-5 F₀ ♂ #9 ^ 05784/60

10 b 6-24-95

37 ♂ +/-

all Agouti

38 ♀ +/-

39 ♀ +/-

40 ♀ +/-

41 ♀ +/+

sac

42 ♂ +/+

43 ♀ +/+

44 ♀ +/+

45 ♂ +/+



C57BL/6

46 ♀ +/+

p2-SF₀ ♂ × C57BL/6 ♀

47 ♂ +/+

7 b

tails

All Agouti

48 ♀ +/+

→ 49 ♂ +/-

mouse with R₁ punch sized 439-453

0/13 positive

50 ♀ +/+

51 ♀ +/+

SAC

52 ♀ +/+

✓ 53 ♀ +/+ SAC

304 M F₀

✓ 54 ♀ +/+ SAC

6 b

✓ 55 ♀ +/+ SAC

tails

✓ 56 ♂ SAC 8/15 +/+

1/6

✓ 57 ♂ SAC 8/15 +/+

✓ 58 ♀ +/30.4m

7/8/63

59 ♂ +/+ SAC
 ✓ 60 ♀ +/- SAC
 61 ♂ +/+
 62 ♀ +/+ SAC
 63 ♀ +/+ SAC
 64 ♂ +/+ SAC
 ✓ 65 ♀ +/- SAC

P2-S F₁
 P2-S F₀ ♂ +/+ x C57BL/6 ♀
 7 b
 (9 total in litter, 2 died)
 All agouti

66 ♀ +/+ A/ SAC
 67 ♂ +/+ A/

P2-S F₁
 P2-S F₀ ♂ +/+ x C57BL/6 ♀
 2 b tails

68 ♂
 69 ♂
 70 ♀ +/304m

304 M F₀ (129 sv)
 3 b tails

71 ♀ B/- A/- +/304m SAC 12/12
 72 ♂ -/- -/- c/c +/304m no good as sire SAC 12/11/95
 73 ♂ B/- A/- SAC tails
 74 ♂ -/- -/- c/c SAC
 75 ♂ B/- A/- +/304m SAC
 76 ♂ -/- -/- c/c SAC
 77 ♀ -/- -/- c/c SAC
 ✓ 78 ♂ b/b a/a +/304m (high copy number) SAC
 79 ♀ b/b A/- SAC

71 (8) / 64

80 ♀ B/- A/- Sac 304 M F₀
 81 ♂ B/- A/- ↓ 10 b
 82 ♀ -/- -/- c/c tails
 83 ♂ B/- a/a +/304M ✓ Sac
 84 ♂ B/- A/- Sac
 85 ♂ -/- -/- c/c ↓
 86 ♂ B/- A/- ? +/304M (single row) Sac 8/22
 87 ♂ b/b a/a Sac
 88 ♀ B/- A/- ↓
 89 ♀ B/- A/-

90 ♀ B/- A/- 304 M F₀
 91 ♂ -/- -/- c/c 15 b
 92 ♂ B/- A/-
 93 ♀ -/- -/- c/c
 94 ♂ B/- A/-
 95 ♂ b/b A/-
 96 ♀ B/- A/- +/304M ✓ Sac (high copy number)
 97 ♂ B/- A/-
 98 ♂ -/- -/- c/c
 99 ♀ B/- a/a +/304M ✓
 100 ♂ B/- A/-
 01 ♂ B/- a/a
 02 ♂ b/b A/-
 03 ♀ B/- A/- +/304M ✓ (high copy #)
 04 ♂ b/b a/a

7/8/65

884

+/+

5

+/+

P2-5

304

886

+/+

+/+

7

+P2-5

8

+P2-5

9

+P2-5

890

+P2-5

1

+/+

2

+/+

3

+/+

4

+/+

+304

+304

+/+

304WK P2-5

♀ 547 (+304WK, B)

♂ 503 (+P2-5)

plug

harvest

blast 123095A

895

a/a

6

7

8

900

1

2

3

d.?

4

PBX-118

F♀ 243 x F♂ 241

96

894*	P25	
5*	+/+	-
6*	+/+	-
7*	+1 P2Br	-
8*	+1 P2Br	+1/304
9*	+1 P2Br	-
900*	+1 P2Br	+1/304
17	+/+	-
2	+1 P2Br	+1/304

304 - P2B
 CBGF, ♀
 plugged by
 304M/P2B 07 392
 harvested
 Blot 010685A

903	+1 P2Br	
4	+/+	+1/304
5	+1 P2Br	-
6	+1 P2Br	-
7	+1 P2Br	-
8	+1 P2Br	-
9	+1 P2Br	-
910	+1 P2Br ← ???	-
1	+1 P2Br	-
2	+1 P2Br	+1/304
3	+/+	+1/304
4	+/+	+1/304

304 - P2B
 CBGF, ♀
 plugged
 by 304M/P2B 07 519
 harvested
 blot 010685B
 blot 011796

8/67

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